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 NEWS 5 FEB 22 The IPC thesaurus added to additional patent databases on STN  
 NEWS 6 FEB 22 Updates in EPFULL; IPC 8 enhancements added  
 NEWS 7 FEB 27 New STN AnaVist pricing effective March 1, 2006  
 NEWS 8 MAR 03 Updates in PATDPA; addition of IPC 8 data without attributes  
 NEWS 9 MAR 22 EMBASE is now updated on a daily basis  
 NEWS 10 APR 03 New IPC 8 fields and IPC thesaurus added to PATDPAFULL  
 NEWS 11 APR 03 Bibliographic data updates resume; new IPC 8 fields and IPC  
 thesaurus added in PCTFULL  
 NEWS 12 APR 04 STN AnaVist \$500 visualization usage credit offered  
 NEWS 13 APR 12 LINSPEC, learning database for INSPEC, reloaded and enhanced  
 NEWS 14 APR 12 Improved structure highlighting in FQHIT and QHIT display  
 in MARPAT  
 NEWS 15 APR 12 Derwent World Patents Index to be reloaded and enhanced during  
 second quarter; strategies may be affected  
 NEWS 16 MAY 10 CA/CAPLUS enhanced with 1900-1906 U.S. patent records  
 NEWS 17 MAY 11 KOREAPAT updates resume  
 NEWS 18 MAY 19 Derwent World Patents Index to be reloaded and enhanced  
 NEWS 19 MAY 30 IPC 8 Rolled-up Core codes added to CA/CAPLUS and  
 USPATFULL/USPAT2  
 NEWS 20 MAY 30 The F-Term thesaurus is now available in CA/CAPLUS  
 NEWS EXPRESS FEBRUARY 15 CURRENT VERSION FOR WINDOWS IS V8.01a,  
 CURRENT MACINTOSH VERSION IS V6.0c(ENG) AND V6.0Jc(JP),  
 AND CURRENT DISCOVER FILE IS DATED 19 DECEMBER 2005.  
 V8.0 AND V8.01 USERS CAN OBTAIN THE UPGRADE TO V8.01a AT  
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\*\*\*\*\* STN Columbus \*\*\*\*\*

FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006

=> file uspatful

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	0.21	0.21

FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

CA INDEXING COPYRIGHT (C) 2006 AMERICAN CHEMICAL SOCIETY (ACS)

FILE COVERS 1971 TO PATENT PUBLICATION DATE: 1 Jun 2006 (20060601/PD)

FILE LAST UPDATED: 1 Jun 2006 (20060601/ED)

HIGHEST GRANTED PATENT NUMBER: US7055175

HIGHEST APPLICATION PUBLICATION NUMBER: US2006117448

CA INDEXING IS CURRENT THROUGH 30 May 2006 (20060530/UPCA)

ISSUE CLASS FIELDS (/INCL) CURRENT THROUGH: 1 Jun 2006 (20060601/PD)

REVISED CLASS FIELDS (/NCL) LAST RELOADED: Feb 2006

USPTO MANUAL OF CLASSIFICATIONS THESAURUS ISSUE DATE: Feb 2006

=> e chang g j j/in

E1	1	CHANG FUO KUO/IN
E2	1	CHANG FWU TSAIR/IN
E3	0 -->	CHANG G J J/IN
E4	1	CHANG GAN CHIEH/IN
E5	2	CHANG GAN HOW/IN
E6	1	CHANG GAO WEI/IN
E7	3	CHANG GAP SOO/IN
E8	5	CHANG GARY/IN
E9	1	CHANG GAUSS/IN
E10	1	CHANG GAVIN/IN

E12 42 CHANG GEE KUNG/IN

=> e chang gwong j j/in

E1 3 CHANG GWO JER/IN  
E2 5 CHANG GWO YANG/IN  
E3 0 --> CHANG GWONG J J/IN  
E4 2 CHANG GWONG JEN J/IN  
E5 2 CHANG GYU HWAN/IN  
E6 2 CHANG H J/IN  
E7 2 CHANG HA Y/IN  
E8 1 CHANG HAE C/IN  
E9 2 CHANG HAE CHOON/IN  
E10 1 CHANG HAE KWUN/IN  
E11 1 CHANG HAE S/IN  
E12 10 CHANG HAE SUNG/IN

=> s e4

L1 2 "CHANG GWONG JEN J"/IN

=> d 11,cbib,1-2

L1 ANSWER 1 OF 2 USPATFULL on STN

2005:188895 Nucleic acid vaccines for prevention of flavivirus infection.

**Chang, Gwong-Jen J**, Fort Collins, CO, UNITED STATES

US 2005163804 A1 20050728

APPLICATION: US 2003-500796 A1 20020404 (10)

WO 2002-US10764 20020404

DOCUMENT TYPE: Utility; APPLICATION.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L1 ANSWER 2 OF 2 USPATFULL on STN

2003:30900 Nucleic acid vaccines for prevention of flavivirus infection.

**Chang, Gwong-Jen J.**, Fort Collins, CO, UNITED STATES

US 2003022849 A1 20030130

APPLICATION: US 2001-826115 A1 20010404 (9)

PRIORITY: US 1998-87908P 19980604 (60)

DOCUMENT TYPE: Utility; APPLICATION.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

=> d 11,cbib,ab,clm,1-2

L1 ANSWER 1 OF 2 USPATFULL on STN

2005:188895 Nucleic acid vaccines for prevention of flavivirus infection.

**Chang, Gwong-Jen J**, Fort Collins, CO, UNITED STATES

US 2005163804 A1 20050728

APPLICATION: US 2003-500796 A1 20020404 (10)

WO 2002-US10764 20020404

DOCUMENT TYPE: Utility; APPLICATION.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The present invention encompasses isolated nucleic acids containing transcriptional units which encode a signal sequence of one flavivirus and an immunogenic flavivirus antigen of a second flavivirus or of a chimeric immunogenic flavivirus antigen comprising sequence from more than one flavivirus. The invention further encompasses a nucleic acid and protein vaccine and the use of the vaccine to immunize a subject against flavivirus infection. The invention also provides antigens encoded by nucleic acids of the invention, antibodies elicited in response to the antigens and use of the antigens and/or antibodies in detecting flavivirus or diagnosing flavivirus infection.

CLM What is claimed is:

1. An isolated nucleic acid comprising a transcriptional unit encoding a signal sequence of a structural protein of a first flavivirus and an immunogenic flavivirus antigen of a second flavivirus, wherein the transcriptional unit directs the synthesis of the antigen.

2. The nucleic acid of claim 1, wherein the signal sequence is a Japanese encephalitis virus signal sequence.

3. The nucleic acid of claim 1, wherein the immunogenic flavivirus antigen is of a flavivirus selected from the group consisting of yellow fever virus, dengue serotype 1 virus, dengue serotype 2 virus, dengue serotype 3 virus, dengue serotype 4 virus, Japanese encephalitis virus, Powassan virus and West Nile virus.

4. The nucleic acid of claim 1, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus and an M protein and an E protein of West Nile virus.

5. The nucleic acid of claim 1, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus and an M protein and an E protein of yellow fever virus.



6. The nucleic acid of claim 1, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus and an M protein and an E protein of St. Louis encephalitis virus.
7. The nucleic acid of claim 1, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus and an M protein and an E protein of Powassan virus.
8. The nucleic acid of claim 1, wherein the antigen is selected from the group consisting of an M protein of a flavivirus, an E protein of a flavivirus, both an M protein and an E protein of a flavivirus, a portion of an M protein of a flavivirus, a portion of an E protein of a flavivirus and both a portion of an M protein of a flavivirus and a portion of an E protein of a flavivirus or any combination thereof.
9. The nucleic acid of claim 8, wherein the antigen is both the M protein and the E protein of a flavivirus.
10. The nucleic acid of claim 1, wherein the nucleic acid is DNA.
11. The nucleic acid of claim 10, comprising a nucleotide sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:19, SEQ ID NO:21 and SEQ ID NO:23.
12. The nucleic acid of claim 1, wherein the transcriptional unit comprises a control sequence disposed appropriately such that it operably controls the synthesis of the antigen.
13. The nucleic acid of claim 12, wherein the control sequence is the cytomegalovirus immediate early promoter.
14. The nucleic acid of claim 1, comprising a Kozak consensus sequence located at a translational start site for a polypeptide comprising the antigen encoded by the TU.
15. The nucleic acid of claim 1 wherein the transcriptional unit comprises a poly-A terminator.
16. A cell comprising the nucleic acid of claim 1.
17. A composition comprising the nucleic acid of claim 1 and a pharmaceutically acceptable carrier.
18. A method of immunizing a subject against infection by a flavivirus, comprising administering to the subject an effective amount of the composition of claim 17.
19. The method of claim 18, wherein the flavivirus antigen is of a flavivirus selected from the group consisting of yellow fever virus, dengue serotype 1 virus, dengue serotype 2 virus, dengue serotype 3 virus, dengue serotype 4 virus, Japanese encephalitis virus, Powassan virus and West Nile virus.
20. The method of claim 18, wherein the antigen is selected from the group consisting of an M protein of a flavivirus, an E protein of a flavivirus, both an M protein and an E protein of a flavivirus, a portion of an M protein of a flavivirus, a portion of an E protein of a flavivirus and both a portion of an M protein of a flavivirus and a portion of an E protein of a flavivirus or any combination thereof.
21. The method of claim 20, wherein the antigen is both the M protein and the E protein of a flavivirus, and wherein a cell within the body of the subject, after incorporating the nucleic acid within it, secretes subviral particles comprising the M protein and the E protein.
22. The method of claim 18, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus, and an M protein and an E protein of West Nile virus.
23. The method of claim 18, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus, and an M protein and an E protein of yellow fever virus.
24. The method of claim 18, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus, and an M protein and an E protein of St. Louis encephalitis virus.
25. The method of claim 18, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus, and an M protein and an E protein of Powassan virus.
26. The method of claim 18, comprising administering the composition to

27. The method of claim 18, wherein the composition is administered via a parenteral route.

28. The nucleic acid of claim 1, wherein the antigen is a St. Louis encephalitis virus antigen.

29. The method of claim 18, wherein the antigen is a St. Louis encephalitis virus antigen.

30. The nucleic acid of claim 1, wherein the antigen is a Japanese encephalitis virus antigen.

31. The method of claim 18, wherein the antigen is a Japanese encephalitis virus antigen.

32. The nucleic acid of claim 1, wherein the antigen is a yellow fever virus antigen.

33. The method of claim 18, wherein the antigen is a yellow fever virus antigen.

34. The nucleic acid of claim 1, wherein the antigen is a dengue virus antigen.

35. The method of claim 18, wherein the antigen is a dengue virus antigen.

36. The nucleic acid of claim 1, wherein the antigen is a West Nile virus antigen.

37. The method of claim 18, wherein the antigen is a West Nile virus antigen.

38. An antigen produced from the nucleic acid of claim 1.

39. A method of detecting a flavivirus antibody in a sample, comprising: (a) contacting the sample with the antigen of claim 38 under conditions whereby an antigen/antibody complex can form; and (b) detecting antigen/antibody complex formation, thereby detecting a flavivirus antibody in the sample.

40. An antibody produced in response to immunization by the antigen of claim 38.

41. A method of detecting a flavivirus antigen in a sample, comprising: (a) contacting the sample with the antibody of claim 40 under conditions whereby an antigen/antibody complex can form; and (b) detecting antigen/antibody complex formation, thereby detecting a flavivirus antigen in a sample.

42. A method of diagnosing a flavivirus infection in a subject, comprising: (a) contacting a sample from the subject with the antigen of claim 38 under conditions whereby an antigen/antibody complex can form; and (b) detecting antigen/antibody complex formation, thereby diagnosing a flavivirus infection in a subject.

43. A method of diagnosing a flavivirus infection in a subject, comprising: (a) contacting a sample from the subject with the antibody of claim 40 under conditions whereby an antigen/antibody complex can form; and (b) detecting antigen/antibody complex formation, thereby diagnosing a flavivirus infection in a subject.

L1 ANSWER 2 OF 2 USPTAFULL on STN

2003:30900 Nucleic acid vaccines for prevention of flavivirus infection.

**Chang, Gwong-Jen J.**, Fort Collins, CO, UNITED STATES

US 2003022849 A1 20030130

APPLICATION: US 2001-826115 A1 20010404 (9)

PRIORITY: US 1998-87908P 19980604 (60)

DOCUMENT TYPE: Utility; APPLICATION.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The present invention encompasses isolated nucleic acids containing transcriptional units which encode a signal sequence of one flavivirus and an immunogenic flavivirus antigen of a second flavivirus. The invention further encompasses a nucleic acid and protein vaccine and the use of the vaccine to immunize a subject against flavivirus infection. The invention also provides antigens encoded by nucleic acids of the invention, antibodies elicited in response to the antigens and use of the antigens and/or antibodies in detecting flavivirus or diagnosing flavivirus infection.

CLM What is claimed is:

signal sequence of a structural protein of a first flavivirus and an immunogenic flavivirus antigen of a second flavivirus, wherein the transcriptional unit directs the synthesis of the antigen.

2. The nucleic acid of claim 1, wherein the signal sequence is a Japanese encephalitis virus signal sequence.

3. The nucleic acid of claim 1, wherein the immunogenic flavivirus antigen is of a flavivirus selected from the group consisting of yellow fever virus, dengue serotype 1 virus, dengue serotype 2 virus, dengue serotype 3 virus, dengue serotype 4 virus, Japanese encephalitis virus, Powassan virus and West Nile virus.

4. The nucleic acid of claim 1, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus and an M protein and an E protein of West Nile virus.

5. The nucleic acid of claim 1, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus and an M protein and an E protein of yellow fever virus.

6. The nucleic acid of claim 1, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus and an M protein and an E protein of St. Louis encephalitis virus.

7. The nucleic acid of claim 1, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus and an M protein and an E protein of Powassan virus.

8. The nucleic acid of claim 1, wherein the antigen is selected from the group consisting of an M protein of a flavivirus, an E protein of a flavivirus, both an M protein and an E protein of a flavivirus, a portion of an M protein of a flavivirus, a portion of an E protein of a flavivirus and both a portion of an M protein of a flavivirus and a portion of an E protein of a flavivirus or any combination thereof.

9. The nucleic acid of claim 8, wherein the antigen is both the M protein and the E protein of a flavivirus.

10. The nucleic acid of claim 1, wherein the nucleic acid is DNA.

11. The nucleic acid of claim 10, comprising a nucleotide sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:19, SEQ ID NO:21 and SEQ ID NO:23.

12. The nucleic acid of claim 1, wherein the transcriptional unit comprises a control sequence disposed appropriately such that it operably controls the synthesis of the antigen.

13. The nucleic acid of claim 12, wherein the control sequence is the cytomegalovirus immediate early promoter.

14. The nucleic acid of claim 1, comprising a Kozak consensus sequence located at a translational start site for a polypeptide comprising the antigen encoded by the TU.

15. The nucleic acid of claim 1 wherein the transcriptional unit comprises a poly-A terminator.

16. A cell comprising the nucleic acid of claim 1.

17. A composition comprising the nucleic acid of claim 1 and a pharmaceutically acceptable carrier.

18. A method of immunizing a subject against infection by a flavivirus, comprising administering to the subject an effective amount of the composition of claim 17.

19. The method of claim 18, wherein the flavivirus antigen is of a flavivirus selected from the group consisting of yellow fever virus, dengue serotype 1 virus, dengue serotype 2 virus, dengue serotype 3 virus, dengue serotype 4 virus, Japanese encephalitis virus, Powassan virus and West Nile virus.

20. The method of claim 18, wherein the antigen is selected from the group consisting of an M protein of a flavivirus, an E protein of a flavivirus, both an M protein and an E protein of a flavivirus, a portion of an M protein of a flavivirus, a portion of an E protein of a flavivirus and both a portion of an M protein of a flavivirus and a portion of an E protein of a flavivirus or any combination thereof.

21. The method of claim 20, wherein the antigen is both the M protein

the subject, after incorporating the nucleic acid within it, secretes subviral particles comprising the M protein and the E protein.

22. The method of claim 18, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus, and an M protein and an E protein of West Nile virus.

23. The method of claim 18, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus, and an M protein and an E protein of yellow fever virus.

24. The method of claim 18, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus, and an M protein and an E protein of St. Louis encephalitis virus.

25. The method of claim 18, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus, and an M protein and an E protein of Powassan virus.

26. The method of claim 18, comprising administering the composition to the subject in a single dose.

27. The method of claim 18, wherein the composition is administered via a parenteral route.

28. The nucleic acid of claim 1, wherein the antigen is a St. Louis encephalitis virus antigen.

29. The method of claim 18, wherein the antigen is a St. Louis encephalitis virus antigen.

30. The nucleic acid of claim 1, wherein the antigen is a Japanese encephalitis virus antigen.

31. The method of claim 18, wherein the antigen is a Japanese encephalitis virus antigen.

32. The nucleic acid of claim 1, wherein the antigen is a yellow fever virus antigen.

33. The method of claim 18, wherein the antigen is a yellow fever virus antigen.

34. The nucleic acid of claim 1, wherein the antigen is a dengue virus antigen.

35. The method of claim 18, wherein the antigen is a dengue virus antigen.

36. The nucleic acid of claim 1, wherein the antigen is a West Nile virus antigen.

37. The method of claim 18, wherein the antigen is a West Nile virus antigen.

38. An antigen produced from the nucleic acid of claim 1.

39. A method of detecting a flavivirus antibody in a sample, comprising: (a) contacting the sample with the antigen of claim 38 under conditions whereby an antigen/antibody complex can form; and (b) detecting antigen/antibody complex formation, thereby detecting a flavivirus antibody in the sample.

40. An antibody produced in response to immunization by the antigen of claim 38.

41. A method of detecting a flavivirus antigen in a sample, comprising: (a) contacting the sample with the antibody of claim 40 under conditions whereby an antigen/antibody complex can form; and (b) detecting antigen/antibody complex formation, thereby detecting a flavivirus antigen in a sample.

42. A method of diagnosing a flavivirus infection in a subject, comprising: (a) contacting a sample from the subject with the antigen of claim 38 under conditions whereby an antigen/antibody complex can form; and (b) detecting antigen/antibody complex formation, thereby diagnosing a flavivirus infection in a subject.

43. A method of diagnosing a flavivirus infection in a subject, comprising: (a) contacting a sample from the subject with the antibody of claim 40 under conditions whereby an antigen/antibody complex can form; and (b) detecting antigen/antibody complex formation, thereby

=> e konishi e/au

E1	2	KONISHI CHIZUKO/AU
E2	2	KONISHI DAISUKE/AU
E3	0 -->	KONISHI E/AU
E4	1	KONISHI EIICHIRO/AU
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E6	4	KONISHI FUMIYA/AU
E7	2	KONISHI FUTOSHI/AU
E8	8	KONISHI GAKU/AU
E9	4	KONISHI GIICHI/AU
E10	2	KONISHI GREGORY A/AU
E11	7	KONISHI HAJIME/AU
E12	3	KONISHI HARUKO/AU

=> e konishi e/in

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E2	2	KONISHI DAISUKE/IN
E3	0 -->	KONISHI E/IN
E4	1	KONISHI EIICHIRO/IN
E5	1	KONISHI FUMIKO/IN
E6	4	KONISHI FUMIYA/IN
E7	2	KONISHI FUTOSHI/IN
E8	8	KONISHI GAKU/IN
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E12	3	KONISHI HARUKO/IN

=> s CMV or CMV-IE

24958 CMV  
24958 CMV  
50642 IE  
914 CMV-IE  
(CMV(W)IE)

L2 24958 CMV OR CMV-IE

=> s l2 and kozak

7920 KOZAK

L3 3918 L2 AND KOZAK

=> s l3 and termination

191047 TERMINATION

L4 3136 L3 AND TERMINATION

=> s l4 and (poly(A))

MISSING TERM 'A))'

The search profile that was entered contains a logical operator followed immediately by a right parenthesis ')'.  
'A))'

=> s l4 and (poly w A)

261228 POLY  
737728 W  
4474137 A  
3 POLY W A  
(POLY(W)W(W)A)

L5 0 L4 AND (POLY W A)

=> s l4 (flavivir? or dengue or japanese encephalitis virus or yellow fever virus or encephalitis virus)

MISSING OPERATOR 'L4 (FLAVIVIR?'

The search profile that was entered contains terms or nested terms that are not separated by a logical operator.  
'L4 (FLAVIVIR?'

=> s l4 and (flavivir? or dengue or japanese encephalitis virus or yellow fever virus or encephalitis virus)

2969 FLAVIVIR?  
2750 DENGUE  
436101 JAPANESE  
8576 ENCEPHALITIS  
101130 VIRUS  
893 JAPANESE ENCEPHALITIS VIRUS  
(JAPANESE(W)ENCEPHALITIS(W)VIRUS)  
259856 YELLOW  
22833 FEVER  
101130 VIRUS  
1232 YELLOW FEVER VIRUS  
(YELLOW(W)FEVER(W)VIRUS)  
8576 ENCEPHALITIS  
101130 VIRUS  
2252 ENCEPHALITIS VIRUS  
(ENCEPHALITIS(W)VIRUS)

=> s 16 and CMV/clm  
1222 CMV/CLM  
L7 33 L6 AND CMV/CLM

=> s 17 and ay<1999  
2811963 AY<1999  
L8 11 L7 AND AY<1999

=> d 18,cbib,clm,kwic,1-11

L8 ANSWER 1 OF 11 USPTAFULL on STN

2001:107871 DNA vaccines against tick-borne **flaviviruses**.

Schmaljohn, Connie S., Frederick, MD, United States

The United States of America as represented by the Secretary of the Army,  
Washington, DC, United States (U.S. corporation)

US 6258788 B1 20010710

**APPLICATION: US 1998-197218 19981120 (9)**

PRIORITY: US 1997-65750P 19971120 (60)

DOCUMENT TYPE: Utility; GRANTED.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A method for inducing a protective immune response to a tick-borne **flavivirus** protein in a mammal, comprising (i) preparing a nucleic acid encoding an antigenic determinant of a tick-borne **flavivirus** prM/E protein operatively linked to a **CMV** promoter operative in cells of a mammal, which nucleic acid is suitable for stably producing the antigenic determinant in a mammal; (ii) coating the nucleic acid in (i) onto carrier particles; (iii) accelerating the coated carrier particles into epidermal cells of the mammal in vivo; and (iv) inducing a protective immune response in said mammal upon exposure to a tick-borne **flavivirus**.
2. The method according to claim 1 wherein the carrier particles are gold.
3. The method according to claim 1 wherein the tick-borne **flavivirus** prM/E protein is selected from the group consisting of Russian spring summer encephalitis prM/E proteins, and Central European encephalitis prM/E proteins.
4. The method according to claim 1 wherein the nucleic acid encodes a protein coding region comprising SEQ ID NO:1.
5. A method for inducing a protective immune response to a tick-borne **flavivirus** protein in a mammal, comprising (i) preparing a nucleic acid encoding an antigenic determinant of a Russian spring summer encephalitis tick-borne **flavivirus** prM/E protein operatively linked to a promoter operative in cells of a mammal, which nucleic acid encodes a protein coding region comprising SEQ ID NO:2 and is suitable for stably producing the antigenic determinant in a mammal; (ii) coating the nucleic acid in (i) onto carrier particles; (iii) accelerating the coated carrier particles into epidermal cells of the mammal in vivo; and (iv) inducing a protective immune response in said mammal upon exposure to a tick-borne **flavivirus**.
6. The method according to claim 1 wherein the nucleic acid encodes a protein coding region comprising SEQ ID NO: 1 and SEQ ID NO:2.
7. A kit for inducing a protective immune response to a tick-borne **flavivirus** protein in a mammal, comprising packaged in association: (a) a nucleic acid encoding an antigenic determinant of a tick-borne **flavivirus** prM/E protein operatively linked to a **CMV** promoter operative in cells of a mammal, which nucleic acid is suitable for stably producing the antigenic determinant in a mammal; (b) one or both of a coating solution and/or components of a coating solution; and (c) carrier particles.
8. The kit of claim 7, wherein the tick-borne **flavivirus** prM/E protein is selected from the group consisting of Russian spring summer encephalitis prM/E proteins, and Central European encephalitis prM/E proteins.
9. The kit of claim 7, wherein the nucleic acid encodes a protein coding region comprising SEQ ID NO:1.
10. The kit of claim 7, wherein the nucleic acid encodes a protein coding region comprising SEQ ID NO:1 and SEQ ID NO:2.
11. A kit for inducing a protective immune response to a tick-borne **flavivirus** protein in a mammal, comprising packaged in association:

summer encephalitis tick-borne **flavivirus** prM/E protein operatively linked to a promoter operative in cells of a mammal which nucleic acid encodes a protein coding region comprising SEQ ID NO:2 and is suitable for stably producing the antigenic determinant in a mammal; (b) one or both of a coating solution and/or components of a coating solution; and (c) carrier particles.

TI DNA vaccines against tick-borne **flaviviruses**  
 AI US 1998-197218 19981120 (9)  
 AB Particle mediated immunization of tick-borne **flavivirus** genes confers homologous and heterologous protection against tick borne encephalitis.  
 SUMM . . . over a wide area of Europe and the former Soviet Union. TBE is most frequently caused by infection with the **flaviviruses** Central European encephalitis (CEE) virus, or Russian spring summer encephalitis (RSSE) virus. These viruses are antigenically and genetically closely related.  
 SUMM . . . of a cytomegalovirus early promoter. We chose the prM and E genes for expression because of earlier reports with other **flaviviruses** which indicated that coexpressed prM and E form subviral particles that are able to elicit neutralizing and protective immune responses.  
 SUMM . . . in adverse side effects to the vaccinated individual. In addition, the invention does not require growth or use of tick-borne **flavivirus**, which may be spread by aerosol transmission and are typically fatal.  
 DRWD . . . those of pWRG1602 described previously (Dimmock, N. J., 1995, Med. Virol. 5: 165) and include a human cytomegalovirus early promoter (**CMV IE** promoter) and intron A, a bovine growth hormone transcription terminator and polyadenylation signal (BGH pA), and a kanamycin resistance gene.  
 DETD In one embodiment, the present invention relates to a DNA or cDNA segment which encodes an antigen from a tick-borne **flavivirus** such as RSSE, CEE, or Langat. More specifically, prM and E genes of CEE were deduced from the CEE viral.  
 DETD . . . were modified around the translation initiation codon (bold type below) to generate sequences with a favorable context for translation initiation (**Kozak**, M., 1989, J. Cell. Biol. 108:229). The forward and reverse primers for RSSE were: 5'GCAGTAGACAGGATGGGTGGTTG3' (SEQ ID NO:3) and 5'GCACAGCCAACCTAAGCTCCCACTCC3'.  
 DETD . . . affecting the ability of the construct to achieve the desired effect, namely induction of a protective immune response against tick-borne **flavivirus** challenge. It is further understood in the art that certain advantageous steps can be taken to increase the antigenicity of. . . by modifying the genetic sequence encoding the protein. It is contemplated that all such modifications and variations of the tick-borne **flavivirus** glycoprotein genes are equivalents within the scope of the present invention.  
 DETD . . . (Konishi, E. et al., 1992, Virology 188:714), or any expression vector such as viral vectors e.g. adenovirus or Venezuelan equine **encephalitis virus** and others known in the art. Preferably, a promoter sequence operable in the target cells is operably linked to the.  
 DETD . . . TBE. Mice have been used extensively as the laboratory model of choice for assessment of protective immune responses to tick-borne **flaviviruses** (Gajdosova, E. et al., 1981, Acta Virol. 25:10; Heinz, F. X. and C. Kunz, 1982, J. Biol. Stand. 10:25; Holzmann, H.).  
 DETD . . . Shope, Yale Arbovirus Research Unit, New Haven, Conn. Cell lines were obtained from the American Type Culture Collection. Central European **encephalitis virus**, strain Hypr, was isolated originally in 1953 from a TBE patient in Czechoslovakia. Russian spring summer **encephalitis virus**, strain Sofjin, was isolated originally in 1937 from a TBE patient from the Far Eastern USSR. Langat virus was isolated.  
 DETD . . . were modified around the translation initiation codon (bold type below) to generate sequences with a favorable context for translation initiation (**Kozak**, M., 1989, J. Cell. Biol. 108:229). The forward and reverse primers for RSSE were: 5'GCAGTAGACAGGATGGGTGGTTG3' (SEQ ID NO:3) and 5'GCACAGCCAACCTAAGCTCCCACTCC3'.  
 DETD . . . have the same control elements; i.e., a human cytomegalovirus early promoter and intron A, and a bovine growth hormone polyadenylation/transcription **termination** signal. However, pWRG7077 does not contain the SV40 virus origin of replication and it has a kanamycin resistance gene rather.  
 DETD Neutralizing antibodies correlate with protective immunity to tick-borne **flaviviruses**, as demonstrated in mice by passive transfer of neutralizing monoclonal antibodies to M and E (Heinz, F. X. et al., . . .  
 DETD . . . 1992, Virology 187:290). Such subviral particles, consisting of heterodimers of prM and E, are also a by product of normal **flavivirus** morphogenesis; i.e., the so-called "slowly sedimenting hemagglutinins" (SHA) (Heinz, F. and C. Kunz, 1977, Acta Virol. 21:308; Mason, P. W. . . and P. W. Mason, 1993, supra). So, although passively transferred

DETD

subsequent **flavivirus** challenge (Buckley, A. and E. A. Gould, 1985, supra; Gould and Buckley, 1986, supra; Heinz, F. X. et al., 1983, . . . al., 1992, Vaccine 10:345). Consequently, although either of our DNA vaccines by itself may be sufficient for immunity to TBE-causing **flaviviruses**, it may be prudent to include both DNAs in a vaccine developed for humans.

1. A method for inducing a protective immune response to a tick-borne **flavivirus** protein in a mammal, comprising (i) preparing a nucleic acid encoding an antigenic determinant of a tick-borne **flavivirus** prM/E protein operatively linked to a **CMV** promoter operative in cells of a mammal, which nucleic acid is suitable for stably producing the antigenic determinant in a . . . of the mammal in vivo; and (iv) inducing a protective immune response in said mammal upon exposure to a tick-borne **flavivirus**.

3. The method according to claim 1 wherein the tick-borne **flavivirus** prM/E protein is selected from the group consisting of Russian spring summer encephalitis prM/E proteins, and Central European encephalitis prM/E. . . .

5. A method for inducing a protective immune response to a tick-borne **flavivirus** protein in a mammal, comprising (i) preparing a nucleic acid encoding an antigenic determinant of a Russian spring summer encephalitis tick-borne **flavivirus** prM/E protein operatively linked to a promoter operative in cells of a mammal, which nucleic acid encodes a protein coding. . . . of the mammal in vivo; and (iv) inducing a protective immune response in said mammal upon exposure to a tick-borne **flavivirus**.

7. A kit for inducing a protective immune response to a tick-borne **flavivirus** protein in a mammal, comprising packaged in association: (a) a nucleic acid encoding an antigenic determinant of a tick-borne **flavivirus** prM/E protein operatively linked to a **CMV** promoter operative in cells of a mammal, which nucleic acid is suitable for stably producing the antigenic determinant in a . . . .

8. The kit of claim 7, wherein the tick-borne **flavivirus** prM/E protein is selected from the group consisting of Russian spring summer encephalitis prM/E proteins, and Central European encephalitis prM/E. . . .

11. A kit for inducing a protective immune response to a tick-borne **flavivirus** protein in a mammal, comprising packaged in association: (a) a nucleic acid encoding an antigenic determinant of a Russian spring summer encephalitis tick-borne **flavivirus** prM/E protein operatively linked to a promoter operative in cells of a mammal which nucleic acid encodes a protein coding. . . .

L8 ANSWER 2 OF 11 USPATFULL on STN

2001:44013 Lentiviral vectors.

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APPLICATION: US 1997-935312 19970922 (8)

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A packaging vector comprising a nucleotide sequence encoding Gag and Pol proteins of a reference lentivirus, said packaging vector differing from said reference lentivirus at least in that (a) it lacks a functional major splice donor site, or its major splice donor site, while functional, differs in sequence from that of said reference lentivirus, and (b) it lacks a functional major packaging signal, which vector, after introduction into a suitable host cell, is capable of causing such cell, either through expression from said vector alone, or through co-expression from said vector and a second vector providing for expression of a compatible envelope protein, to produce packaging vector particles comprising functional Gag and Pol proteins and having a normal or a pseudotyped envelope, where said particles are free of the RNA form of said packaging vector as a result of (b) above, where said cell, as a result of said expression or co-expression, produces particles encapsulating the RNA form of a transducing vector possessing a compatible and functional packaging signal if said transducing vector is introduced into said cell, where said reference lentivirus is a human or simian immunodeficiency virus.

2. The packaging vector of claim 1 in which the reference lentivirus is HIV-1.

3. The packaging vector of claim 1 in which the reference lentivirus is HIV-2.

4. The packaging vector of claim 1 in which the reference lentivirus is SIV.



proteins.

6. The packaging vector of claim 1 which does not encode a functional envelope protein.

7. The packaging vector of claim 1 wherein the major splice donor site of said vector differs in sequence from that of any lentivirus major splice donor site sufficiently so that said major splice donor site is not a potential site for homologous recombination between said packaging vector and any HIV or SIV.

8. The packaging vector of claim 1 which comprises a sequence encoding a lentivirus Env proteins.

9. The packaging vector of claim 1 which comprises a sequence encoding the VSV-G envelope protein.

10. The packaging vector of claim 1 which further differs from said reference lentivirus in that at least portions of at least one gene selected from the group consisting of the env, vpr, vif, and vpu genes of said reference lentivirus is or are deleted.

11. The packaging vector of claim 1 which lacks the native primer binding site of said reference lentivirus.

12. The packaging vector of claim 1 which lacks the native polypurine tract of said reference lentivirus.

13. The packaging vector of claim 1 which lacks a functional nef gene.

14. The packaging vector of claim 1 which further differs from said lentivirus in that the 5' LTR has been modified.

15. The packaging vector of claim 1 in which the 5'LTR is a chimera of a lentivirus LTR and a CMV enhancer/promoter.

16. The packaging vector of claim 1 comprises a tat gene and a TAR sequence.

17. The packaging vector of claim 1 which comprises a rev gene and an RRE element.

18. The packaging vector of claim 1 which further differs from the reference lentivirus in that at least a portion of the tat gene and the TAR sequence are deleted.

19. The packaging vector of claim 1 which further differs from the reference lentivirus in that at least a portion of the env gene and the RRE element are deleted.

20. A packaging cell which comprises the packaging vector of claim 1 and is suitable for production of packaging or transducing vector particles.

21. A method of producing a transducing vector comprising a remedial gene, in the form of an infectious particle, which comprises (a) transfecting a cell with a packaging vector according to claim 1, and, if said packaging vector does not itself provide for expression of a compatible envelope protein, a pseudotyping vector which does provide expression, so said cell is capable of producing packaging vector particles, (b) transfecting said cell with a transducing vector comprising said remedial gene, and a functional packaging signal, but which by itself is incapable of causing a cell to produce transducing vector particles, and (c) causing the cell to produce infectious transducing vector particles comprising said transducing vector in RNA form, said Gag and Pol proteins, and said envelope protein.

22. A kit comprising a packaging vector according to claim 1 and a transducing vector comprising a functional and compatible packaging signal, said transducing vector being incapable by itself of causing a cell transfected by said transducing vector to encapsulate the RNA form of said transducing vector into a transducing vector particle.

23. The packaging vector of claim 1 in which the major splice donor site is a modified RSV major splice donor site corresponding to the splice donor site included in SEQ ID NO:9 and SEQ ID NO:10.

24. The packaging vector of claim 1 where said major splice donor site is functional but differs in sequence from that of all HIV and SIV lentivirus splice donor sites.

25. The packaging vector of claim 1 which lacks a functional major splice donor site.

26. The packaging vector of claim 1 where its major splice donor site, while functional, differs in sequence from that of said reference lentivirus sufficiently so that homologous recombination between said packaging vector and said reference lentivirus at said splice donor site is not detectable.

27. The vector of claim 1, wherein at least a portion of the env gene of said reference lentivirus is deleted.

28. The packaging vector of claim 7 wherein the major splice donor site of said vector is substantially identical to the RSV splice donor site.

29. The cell of claim 20, which further comprises a pseudotyping vector.

30. The cell of claim 20 which further comprises a transducing vector which by itself is incapable of coding for expression of infectious transducing vector particles, but which cell, as a result of the expression of genes of said packaging vector, packages the RNA form of said transducing vector into infectious transducing vector particles.

31. The cell of claim 20 where said transducing vector further comprises a remedial gene.

32. The cell of claim 20 wherein packaging is inducible.

33. The kit of claim 22, said packaging vector comprising a gene encoding a compatible envelope protein.

34. The kit of claim 22, further comprising a pseudotyping vector comprising a gene encoding a non-lentiviral envelope protein incorporatable into said particles.

35. The packaging vector of claim 26 in which the absence of detectable homologous recombination is demonstrated by failure to detect replication-competent virus transfecting human TE671 cells with the packaging vector, co-culturing the TE671 cells with the human lymphoma cell line MT4 for two months, and determining, by immunohistochemical methods, whether the MT4 cells are producing HIV-1 proteins.

36. The vector of claim 27 in which the deletion is a frame shift mutation.

37. The vector of claim 27 in which two nucleotides of the env gene are deleted.

38. The vector of claim 27 in which 28 nucleotides of the env gene are deleted.

39. The vector of claim 27 in which the deletion is one achievable by Bal31 digestion at the unique NheI site in the env gene of wild-type HIV strain pNL4-3 or at the corresponding position in another reference lentivirus.

40. The vector of claim 35 where the presence of replication-competent virus is detected by determining by immunohistochemical methods whether the MT4 cells are producing HIV-1 proteins.

AI US 1997-935312 19970922 (8)

SUMM . . . present in the M-MuLV LTR is quite weak compared with other viral promoters such as the human cytomegalovirus immediate early (CMV-IE) enhancer/promoter. In order to increase expression of the genes carried on the retroviral vector, internal promoters possessing stronger activities than. . .

SUMM . . . consisting of human immunodeficiency virus type 1, human immunodeficiency virus type 2, feline immunodeficiency virus, simian immunodeficiency virus, visna-maedi, caprine arthritis-encephalitis virus, equine infectious anemia virus, and bovine immune deficiency virus.

SUMM . . . consisting of human immunodeficiency virus type 1, human immunodeficiency virus type 2, feline immunodeficiency virus, simian immunodeficiency virus, visna-maedi, caprine arthritis-encephalitis virus, equine infectious anemia virus, and bovine immune deficiency virus. Thus, the recombinant lentivirus may be recombinant HIV-1, HIV-2, SIV, or. . . other embodiments, the lentiviral vector further comprises plasmid DNA selected from the group consisting of pHP-1, pHP-dl.2 and pHP-dl.28, pHP-VSVG, pHP-CMV, pHP-CMVdel.TAR/SD, pHP-CMV-EF1 $\alpha$  intron, and pHP-EF.

SUMM . . . cell and/or cell line contains a transducing vector is selected from the group consisting of pTV $\Psi$ , pTV $\Psi$ 100, pTV $\Psi$ 140, pTV $\Psi$ .nlacZ, and pTV $\Psi$ -CMV-nlacZ-hyg-dl.SmaI, pTV $\Delta$ , pTV $\Delta$ -X, pTV $\Delta$ -CMV-X, pTV $\Delta$ -CMVnlacZ, pTV $\Delta$ SVneo,

... consisting of human immunodeficiency virus type 1, human immunodeficiency virus type 2, feline immunodeficiency virus, simian immunodeficiency virus, visna-maedi, caprine arthritis-**encephalitis virus**, equine infectious anemia virus, and bovine immune deficiency virus. Thus, the attenuated virus may be an attenuated HIV-1, attenuated HIV-2, . . .

FIG. 1D provides simplified schematic illustrations of three heterologous enhancer/promoter inserts (human **CMV IE(a)**, human **CMV IE(b)**, and Mo-MLV).

. . . agent of the human acquired immunodeficiency syndrome (AIDS); visna-maedi, which causes encephalitis (visna) or pneumonia (maedi) in sheep, the caprine arthritis-**encephalitis virus**, which causes immune deficiency, arthritis, and encephalopathy in goats; equine infectious anemia virus, which causes autoimmune hemolytic anemia, and encephalopathy. . .

. . . (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and **termination** and polyadenylation signals. In some embodiments, "expression vectors" are used in order to permit pseudotyping of the viral envelope proteins.

In the present invention, various transducing vectors may be used, including pTV $\Psi$ , pTV $\Psi$ 100, pTV $\Psi$ 140, pTV. $\Psi$ .nlacZ, and pTV $\Psi$ **CMV**-nlacZ-hyg-dl.SmaI, pTV $\Delta$ , pTV $\Delta$ -X, pTV $\Delta$ **CMV**-X, pTV $\Delta$ CMVnlacZ, pTV $\Delta$ SVneo, pTV $\Delta$ SVhyg, pTV $\Delta$ **CMV**-GFP, pTV $\Delta$ **CMV**-nlacZ, and pTV $\Delta$ **CMV**-nlacZ-hyg. However, it is not intended that the present invention be limited to these specific transducing vectors. For example, the "pTV $\Delta$ -X," . . .

. . . However, enhancer elements can exert their effect even when located 3' of the promoter element and the coding region. Transcription **termination** and polyadenylation signals are located 3' or downstream of the coding region.

As used herein, the term "transcription unit" refers to the segment of DNA between the sites of initiation and **termination** of transcription and the regulatory elements necessary for the efficient initiation and **termination**. For example, a segment of DNA comprising an enhancer/promoter, a coding region and a **termination** and polyadenylation sequence comprises a transcription unit.

. . . which facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements are splicing signals, polyadenylation signals, **termination** signals, etc. (defined infra).

Efficient expression of recombinant DNA sequences in eukaryotic cells requires expression of signals directing the efficient **termination** and polyadenylation of the resulting transcript. Transcription **termination** signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly A site" or "poly A sequence" as used herein denotes a DNA sequence which directs both the **termination** and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly. . . signal. The SV40 poly A signal is contained on a 237 bp Bam HI/Bcl I restriction fragment and directs both **termination** and polyadenylation (J. Sambrook et al., supra, at 16.6-16.7).

. . . was not markedly affected by these mutations. By mutating the tat gene, it was also found that the recombinant LTRs (**CMV-IE-HIV-LTR**) exhibited increased basal levels of promoter activity which could support virus replication without Tat (L. -J. Chang, and C. Zhang, . . .

LTR mutants with kB/Spl or Spl deletion and **CMV-IE** enhancer/promoter insertion have been shown to replicate with delayed kinetics in human lymphocyte culture, including primary PBLs (peripheral blood lymphocytes). . .

. . . were generated using the LTR mutant constructs which exhibited enhanced transcriptional activity after inserting heterologous enhancer elements. The recombinant LTR (**CMV-IE-HIV-LTR**), which has been shown to exhibit increased basal level of promoter activity, can support HIV-1 replication without Tat (L. -J. . .

. . . present invention, it was determined that the tat-C mutant is more defective than the tat-A and -B mutants, and the dl.Spl/**CMV** tat-B double mutant is more defective than the dl.Spl/**CMV** LTR mutant or the dl.Spl/**CMV** tat-A double mutant reported previously (L. -J. Chang and C. Zhang, Virol., 211:157-169 [1995]). The dl.Spl/**CMV** tat-B double mutant infects human lymphoid cell lines with delayed kinetics and exhibited reduced cytopathic effects.

. . . PBLs poorly and replicated in primary macrophage culture with reduced kinetics. Based on these results, these already attenuated HIV-1 constructs, dl.Spl/**CMV** tat-B and dl.Spl/**CMV** tat-C, were chosen for

DETD . . . LTR/tat mutants were further characterized in human lymphoid cell culture. The tat-A or tat-B LTR double mutants (Spl deleted and **CMV-IE** enhancer inserted) infected human MT4 cells with slightly reduced cytopathic effects. Further, these mutants exhibited delayed replication kinetics when compared with wild-type HIV-1. On the other hand, when cells were infected with the tat-C LTR mutant (Spl/**CMV** mutant), the cytopathic effect was not so apparent and interestingly, the infected culture recovered rapidly and a persistent infection was.

DETD . . . HIV-1 Infected Cultures

Cell Line/Virus	% Viability	Doubling Time
	(±5%)	(±2 hrs)
MT4/(mock)	88	40
MT4/WT (acute)	0	---a
MT4/tat-A (dl.Spl/ <b>CMV</b> )	0	--
MT4/tat-B (Dl.Spl/ <b>CMV</b> )	0	--
MT4/tat-C (chr.1)	97	35
MT4/tat-C (chr.2)	86	32
AA2/WT (chr.)	73	n.d.b
Molt3/WT (chr.)	80	n.d.

a "--," No. . . .

DETD Five additional HP constructs were also made ("pHP-VSVG," lipHP-**CMV**," "pHP-EF," "pHP-CMVdel.TAR/SD," and "pHP-**CMV**-EF1 $\alpha$ -intron"), each with additional changes (See, FIG. 7). pHP-VSVG was derived from pHP-1, with the HIV-1 env gene being replaced by the VSV-G gene and containing either wild-type (pHP-NVSV-G) or mutated (pHP-VSV-G) vpr and tat genes. pHP-**CMV** was derived from pHP-1, with the promoter being replaced by the cytomegalovirus immediate early promoter (**CMV-IE**) and the tat, rev, env, vpr and vpu genes deleted. pHP-CMVdel.TAR/SD was derived from pHP-**CMV**, with the TAR and RSV RD deleted. pHP-**CMV**-EF1 $\alpha$ -intron was derived from pHP-CMVdel.TAR/SD, with an insertion of the EF1 $\alpha$ -intron between the promoter and the Gag AUG. pHP-EF was derived from pHP-**CMV**, by replacing the **CMV-IE** promoter and the synthetic SD site with the human elongation factor 1 $\alpha$  (EF1 $\alpha$ ) enhancer plus intron. The TAR sequence was. . . transduction efficiency in nondividing culture. In other experiments, the intron-containing EF1 $\alpha$  was shown to be a stronger promoter than the **CMV-IE** promoter.

DETD . . . of Gag-Pol (e.g., pHP-1, pHP-1del, and pHP-VSVG), as well as vectors that do not express detectable amounts of Gag-Pol (e.g., pHP-**CMV** and its derivatives).

DETD . . .  $\Psi$  signals were cloned into the pTV $\Psi$  vector as shown in FIG. 8, which is comprised of two recombinant LTRs ("dl.kB-**CMV**/HIV-TAR"), the PBS and 5' leader sequences, an SV40-driven neo resistance gene, and the 3' PPT.

DETD . . . an additional gag sequence and an RRE element, were cloned into pTV $\Psi$ 140. One such example is shown in FIG. 9A (pTV $\Psi$ +**CMV**-nlacZ-hyg). Again, the pTV $\Psi$ + was not packaged efficiently, indicating the splice donor site and Gag AUG mutations in pTV $\Psi$ 100 and pTV $\Psi$ 140.

DETD Three additional pTVA vectors were also constructed, each containing a different reporter gene: **CMV**-GFP (green fluorescent protein, pTVA**CMV**-GFP), **CMV**-nlacZ (pTVA**CMV**-nlacZ) and **CMV**-nlacZ-hyg (pTVA**CMV**-nlacZ-hyg), as illustrated in FIG. 8 (See, FIG. 8, constructs 5 and 6, as well as FIG. 9B). The production of VSV-G pseudotyped vector was tested with pTVA**CMV**-nlacZ. TE671 cells transduced with the VSV-G pseudotyped pTVA**CMV**-nlacZ vector stained strongly by X-gal and exhibited nuclear  $\beta$ -galactosidase activity. The pTVA**CMV**-nlacZ-hyg and pTVA**CMV**-GFP did not express the reporter genes efficiently, whereas pTVA**CMV**-nlacZ did. These transducing vectors were further characterized using dividing and nondividing tissue culture models and a small animal model.

DETD . . . by recombination. Thus, pHP-1 provides an excellent HIV DNA vector. pHP-1 was constructed as follows. First, the Tat-responsive enhancer promoter **CMV**-TATA-TAR fragment (approximately 400 bp) was isolated from dl.kB/Spl-**CMV**-TATA-TAR HIV (Chang et al., J. Virol. 67:743 [1993]) by BbrpI-HindIII digestion, and cloned into EcoRV-BamHI digested pSP72 (Promega) via a linker providing HindIII and BamHI cohesive sites which contains a modified gag AUG with **Kozak** translation initiation context and a major splice donor site of Rous sarcoma virus. This linker was formed by annealing the following oligonucleotides: 5'-AGCTTGGTCGCCCGGTGGATCAAGACCGGTAGCCGTCATAAAGGTGAT TTCGTCG-3' (SEQ ID NO:9) and 5'-GATCCGACGAAATCACCTTTATGACG GCTACCGGTCTTGATCCACCGGGCGACCA-3' (SEQ ID NO:10). This first subclone was called pSP-**CMV**-TAR-SD.

DETD . . . [SEQ ID NO:12]). The PCR product was digested with BamHI-SphI (.about.660 bp) and this fragment was ligated with BamHI-SphI digested pSP-**CMV**-TAR-SD to obtain pSP-**CMV**-TAR-SD-dl.gag.

DETD . . . the poly-A minus subclone pHP-dl.pA was constructed by ligating

from pSP-**CMV**-TAR-SD-dl.gag (contains the promoter-TAR-SD-dl.gag), a 7922 bp SphI-XhoI fragment (dl.gag-pol-env-gpt) of pNLgpt, and a plasmid vector backbone provided by EcoRV-XhoI digested. . . .

DETD Lastly, pHP-1 was made by the following ligation: NotI-XhoI (9059 bp) of pHP-dl.pA containing dl.**CMV**-TATA-TAR-SD-gag-pol-env-gpt, a 422 bp poly-A site from XhoI-PstI digested pREP9 (Invitrogen), and NotI-PstI digested pBS-KS(-). The sequence of pHP-1 (12,494 kb). . . .

DETD As described in more detail below, five other HP constructs were made, pHP-VSVG, three pHP-**CMV** derivatives, and pHP-EF, each with additional changes (See, FIG. 7). pHP-VSVG was derived from pHP-1, with the HIV-1 env gene. . . . by the VSV-G gene, and with wild-type vpr and tat, or the vpr and tat genes mutated by site-specific mutagenesis. pHP-**CMV** was derived from pHP-1 with the promoter being replaced by the cytomegalovirus immediate early promoter (**CMV-IE**) and the tat, rev, env, vpr and vpu deleted. pHP-**CMVdel**.TAR/SD was derived from pHP-**CMV**, with the TAR and RSV RD deleted. pHP-**CMV**-EF1 $\alpha$ -intron was derived from pHP-**CMVdel**.TAR/SD, with an insertion of the EF1 $\alpha$ -intron between the promoter and the Gag AUG. pHP-EF was derived from pHP-**CMV** by replacing the **CMV-IE** promoter and the synthetic SD site with the human elongation factor 1 $\alpha$  (EF1 $\alpha$ ) enhancer plus intron. It also contains an. . . . the vector transduction efficiency in non-dividing cultures. The intron-containing EF1 $\alpha$  has been shown to be a stronger promoter than the **CMV-IE** promoter. These constructs were tested for their expression of HIV-1 proteins. pHP-VSVG did not express HIV-1 proteins unless the Tat. . . .

DETD Both packaging constructs (i.e., pHP-1 and pHP-VSVG) used a recombinant **CMV**/HIV-LTR as promoter and a synthetic major splice donor site. No sequence homology was observed with the HIV-1 genome between TAR. . . .

DETD These experiments showed that pHP-**CMV** and pHP-EF do not express Gag-Pol proteins at high efficiencies, indicating that the pHP-1-derived vectors have important viral sequences that. . . .

DETD . . . vpr and tat genes. It was constructed by combining the following four pieces of DNA fragments: 1) the recombinant LTR (dl.kB/Spl-**CMV**-TATA-HIV-TAR) gag-pol from NotI to EcoRI fragment of pHP-1; 2) a fragment from HIV-1 with deletion in the C-terminal of Vpr. . . .

DETD pHP-**CMV**.

DETD This clone was derived from pHP-1, with the 5' recombinant LTR replaced by a **CMV-IE** enhancer-promoter and the entire env, tat, vpu, rev, vpr, nef deleted, but with the vif gene remaining intact. This clone. . . .

DETD pHP-**CMV**-del.TAR/SD:

DETD This clone is the same as pHP-**CMV** except that the 5' TAR and splice donor site are deleted. This construction was made by ligating the following two fragments: 1) a 702 bp fragment of MluI-BamHI digested pcDNA3.1Zeo(+) containing the **CMV** enhancer; and 2) the vector containing MluI-BamHI digested pHP-**CMV** which has deleted TAR and contains the RSV splice donor site.

DETD pHP-**CMV**-EF1 $\alpha$ -intron.

DETD This clone is similar to pHP-**CMV**-del.TAR/SD but with an intron from human EF-1 $\alpha$  gene inserted between the **CMV** promoter and the gag AUG. It was made by ligating the following three DNA fragments: 1) pHP-1 BamHI-EcoRI fragment containing. . . . gag-pol and vif; 2) the MluI-EcoRI of pcDNA3zeonlacZ-RRE containing the vector backbone of pcDNA3.1Zeo(+), HIV-1 RRE and part of the **CMV** promoter; and 3) the rest of the **CMV** enhancer promoter was obtained from BamHI-MluI digested pcDNA3zeoHGHP2EF, a pcDNA3zeo3.1(+) vector containing EF1 $\alpha$  intron and the human growth hormone gene. . . .

DETD Four additional packaging vectors, pHP-**CMV** derivatives, and pHP-EF, were constructed as shown in FIG. 7. The heterologous enhancer/promoters in these vectors may express high levels. . . . of GFP is much improved when an intron sequence was inserted in front of the GFP gene. All of the pHP-**CMV** derivatives were tested, and found to be inefficient in synthesizing HIV proteins, indicating that the pHP-1 and pHP-VSVG derivatives are. . . .

DETD . . . sequence. Sequences in gag-pol and env genes are deleted and the major SD and the gag AUG are mutated. A **CMV**-driven reporter gene cassette such as the **CMV-IE**-nlacZ-IRES-hyg from the pTVA-nlacZ-hyg vector is inserted in the nef ORF of the HIV-2 and the SIV vectors. The 3' LTR. . . .

DETD Internal **CMV-IE** in pTV $\Delta$ CMVnlacZ Promoter Exhibits Higher Promoter Activity Than Native **CMV-IE**

DETD In this Example, the expression of the reporter lacZ gene from the pTV- $\Delta$ CMVnlacZ was compared with pcDNAnlacZ (i.e., **CMV-IE** promoter-driven), 48 hours after transfection of TE671 cells. TE671 cells were transfected with 5  $\mu$ g of pcDNA3-nlacZ or pTV $\Delta$ CMVnlacZ, as. . . .

DETD . . . sequences near the 5' end of the PPT of HIV-1, the product was then ligated with a SalI-KpnI fragment containing **CMV**-nlacZ sequence from pcDNAzeo-nlacZ. pcDNAzeo-nlacZ was generated by inserting nlacZ of pSP72nlacZ into pcDNA3.1zeo(+).

# Production of High-Titer HIV-1 Derived Vectors

Packaging Construct	Pseudotyped Envelope	Transducing Vector	Addi- tional Genes	RT (cpm/ $\mu$ l)	Titer (cfu/ml)
pNL4-3	pHEF-10 <sup>4</sup>	pTV $\Delta$ <b>CMV</b>		1.1 $\times$ 10 <sup>5</sup>	7.9 $\times$ 10 <sup>4</sup>
pNL-4-3	VSVG	nlacZ			
pNL-4-3	pHEF-VSVG	pTV $\Delta$ <b>CMV</b> -nlacZ-hyg-dl.SmaI		7.9 $\times$ 10 <sup>4</sup>	24
PHP-1	pHEF-10 <sup>5</sup>	pTV $\Delta$ <b>CMV</b> pCEP-		3.7 $\times$ 10 <sup>4</sup>	2.5 $\times$ 10 <sup>4</sup>
PHP-1	VSVG	nlacZ	tat		
PHP-1	pHEF-VSVG	pTV $\Delta$ <b>CMV</b> pCEP-nlacZ-hyg-dl.SmaI		3.1 $\times$ 10 <sup>4</sup>	100
PHP-2dl.2	pHEF-10 <sup>5</sup>	pTV $\Delta$ <b>CMV</b> pCEP-		3.9 $\times$ 10 <sup>4</sup>	1.7 $\times$ 10 <sup>4</sup>
PHP-1dl.2	VSVG	nlacZ	tat		
PHP-1dl.2	pHEF-VSVG	pTV $\Delta$ <b>CMV</b> pCEP-nlacZ-hyg-dl.SmaI		3.6 $\times$ 10 <sup>4</sup>	90

DETD . . . 4

## Detection of Replication-Competent HIV (RCV)

Packaging Construct	Pseudotyped Envelope	Trans- ducing Vector	Addi- tional Genes	Days After Co-Culture	
pNL4-3 (Control)	pHEF-VSVG	pTV $\Delta$ <b>CMV</b> -nlacZ		8	60#
PHP-1	pHEF-VSVG	pTV $\Delta$ <b>CMV</b> pCEP-nlacZ		28	
PHP-1dl.2	pHEF-VSVG	pTV $\Delta$ <b>CMV</b> pCEP-nlacZ		28	
PHP-1dl.28	pHEF-VSVG	pTV $\Delta$ <b>CMV</b> pCEP-nlacZ		28	

\*Results of rapid cell death and loss of MT4 cells.

`+ to ++++', approximately 10. . .

DETD . . . (BAS1000). The results are shown in FIG. 14: Lane 1, control MT4; lane 2 & 3, MT4 chronically infected with dl.Spl **CMV** tat-C; lane 4, MT4 acutely infected with WT HIV-1; lane 5, C8166 chronically infected with WT HIV-1; lane 6, MT4 chronically infected with dl.Spl **CMV** tat-B; lane 7, AA2 chronically infected with dl.Spl **CMV** tat-C. . . 15. The packaging vector of claim 1 in which the 5'LTR is a chimera of a lentivirus LTR and a **CMV** enhancer/promoter.

L8 ANSWER 3 OF 11 USPATFULL on STN

2000:7195 Method for stimulating an immune response utilizing recombinant alphavirus particles.

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US 6015694 20000118

**APPLICATION: US 1997-931869 19970916 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A method of stimulating in an animal an immune response to an antigen, comprising infecting susceptible animal target cells with recombinant alphavirus particles comprising a vector which directs the expression of at least one antigen or modified form thereof in target cells infected with the alphavirus, wherein said antigen or modified form thereof stimulates an immune response within an animal, and wherein said recombinant alphavirus particles are obtained from an alphavirus packaging cell comprising a stably transformed expression cassette which expresses an alphavirus structural protein, which, after introduction of an alphavirus vector construct, produces recombinant alphavirus particles.

2. The method according to claim 1 wherein said antigen is a viral antigen.

3. The method according to claim 2 wherein said viral antigen is obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, HBV, HIV, HSV, FeLV, FIV, HTLV-1, HTLV-2, and **CMV**.

4. The method according to claim 2 wherein said viral antigen is

5. The method according to claim 1 wherein said antigen is a tumor antigen.
6. The method according to claim 1 wherein said antigen is obtained from a bacteria, parasite or fungus.
7. The method according to claim 1, wherein said alphavirus vector construct is introduced into said packaging cell by transfecting a eukaryotic layered vector initiation system or an alphavirus vector construct RNA into said packaging cell.
8. The method according to claim 1, wherein said alphavirus vector construct is introduced into said packaging cell by infecting said packaging cell with a recombinant alphavirus particle.
9. A method of stimulating in an animal an immune response to an antigen, comprising infecting susceptible animal target cells with recombinant alphavirus particles which direct the expression of at least one antigen or modified form thereof in target cells infected with the alphavirus, wherein said antigen or modified form thereof stimulates an immune response within an animal, and wherein said recombinant alphavirus particles are free from recombinant alphavirus particles that can initiate a productive infection that yields infective alphavirus particles.
10. A method according to any one of claims 1 or 9 wherein the target cells are infected within said animal.
11. A method according to any one of claims 1 or 9 wherein the expressed antigen elicits an immune response selected from the group consisting of a cell-mediated immune response, a HLA class I-restricted immune response, and a HLA Class II-restricted immune response.

**AI US 1997-931869 19970916 (8)**

SUMM . . . transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**.

SUMM . . . transcription of the subgenomic fragment is reduced, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**.

SUMM . . . transcription of the subgenomic fragment is reduced, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**.

SUMM . . . cell, the vector construct being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**.

SUMM . . . the vector construct being capable of expressing a heterologous ribonucleic acid sequence, and a 3' DNA sequence which controls transcription **termination**.

SUMM . . . from cDNA is selected from the group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40 promoter, and the **CMV** promoter. Within further embodiments, the eukaryotic layered vector initiation systems further comprise a polyadenylation sequence.

SUMM . . . group consisting of influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and **CMV**. Within one preferred embodiment, the heterologous sequence obtained from HPV encodes a protein selected from the group consisting of E5, . . .

SUMM . . . are complementary to influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and **CMV** sequences.

SUMM . . . SC distal, SV40, heat shock protein 65, heat shock protein 70, Py, RSV, BK, JC, MuLV, MMTV, alphavirus junction region, **CMV** and VA1RNA.

DETD . . . reduce viral transcription of the subgenomic fragment, an alphavirus RNA polymerase recognition sequence, and a 3t sequence which controls transcription **termination**. The vector may also include nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, . . .

DETD . . . sequence encoding one or more proteins, said proteins preferably comprising alphavirus structural protein(s). Optionally, the expression cassette may include transcription **termination**, splice recognition, and polyadenylation addition sites. Preferred promoters include the **CMV**, MMTV, MoMLV, and adenovirus VA1RNA promoters. In addition, the expression cassette may contain selectable markers such as Neo, SV2 Neo, . . .

DETD . . . promoters, such as, for example, the  $\beta$ -galactosidase promoter, trpE promoter, lacZ promoter, T7 promoter, T3 promoter, SP6 promoter, SV40 promoter, **CMV** promoter, and MoMLV LTR.

DETD 4. The **CMV** H301 Gene

DETD . . . the human immunoglobulin heavy-chain binding protein, and a synthetic sequence of at least 15 bps corresponding in part to the **Kozak** consensus sequence for efficient translational initiation.

translation initiation can also. . .

DETD As noted above, the alphavirus cDNA vector construct also includes a 3' sequence which controls transcription **termination**. A representative example of such a sequence is set forth in more detail below in Examples 2 and 3.

DETD . . . vector may be selected to hybridize to any pathogen-specific RNA, for instance target cells infected by pathogens such as HIV, **CMV**, HBV, HPV and HSV.

DETD . . . that any truncation leave intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational **termination** codons may be introduced downstream of the immunogenic region. Insertion of **termination** codons will prematurely terminate protein expression, thus preventing expression of the transforming portion of the protein.

DETD . . . "HCV", respectively), Human Papiloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, TTLV I, HTLV II and Cytomegalovirus ("**CMV**"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which. . .

DETD . . . cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**. Briefly, such eukaryotic layered vector initiation systems provide a two-stage or "layered" mechanism which controls expression of heterologous nucleotide sequences. . . promoter which is capable of initiating the 5' synthesis of RNA from cDNA (e.g., a 5' promoter), a 3' transcription **termination** site, as well as one or more splice sites and/or a polyadenylation site, if desired. Representative promoters suitable for use. . . Mouse polyoma virus promoter ("Py"), rous sarcoma virus ("RSV"), BK virus and JC virus promoters, MMTV promoter, alphavirus junction region, **CMV** promoter, Adenovirus VA1RNA, rRNA promoter, tRNA methionine promoter, CaMV 35S promoter, nopaline synthetase promoter, and the lac promoter. The second. . .

DETD . . . example, vector systems derived from viruses of the following families: Picornaviridae (e.g., poliovirus, rhinovirus, coxsackieviruses), Caliciviridae, Togaviridae (e.g. alphavirus, rubella), **Flaviviridae** (e.g., yellow fever), Coronaviridae (e.g., HCV, TGEV, IBV, MHV, BCoV), Bunyaviridae, Arenaviridae, Retroviridae (e.g., RSV, MoMLV, HIV, HTLV), hepatitis delta. . .

DETD . . . transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**. Within various embodiments, the viral junction region may be modified, such that viral transcription of the subgenomic fragment is merely. . .

DETD . . . auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV HTLV I, HTLV II, **CMV**, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease.

DETD . . . cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**. Within one embodiment, such constructs may be constructed of the following ordered elements: a 5' eukaryotic promoter capable of initiating. . . alphavirus non-structural proteins, a viral junction region, a heterologous sequence, an alphavirus RNA polymerase recognition sequence, and a 3' transcription **termination**/polyadenylation signal sequence. Such alphavirus cDNA expression vectors may also include intervening sequences (introns), which are spliced from the pre-RNA in. . .

DETD . . . occurs from the pKSSINBVdLJR construct. Junction region changes at Sindbis nts 7600 and 7602 are downstream of the NSP 4 **termination** codon and upstream of the structural proteins initiation codon.

DETD . . . are observed among the eight alphaviruses sequenced (Semliki Forest virus, Middleburg virus, Ross River virus, O'Nyong Nyong virus, Eastern Equine **Encephalitis virus**, Western Equine **Encephalitis virus**, and Venezuelan Equine **Encephalitis virus**) are given below:

DETD In order to insert the luciferase gene into the pVGELVIS-SINBV vector, the SV40 intron and transcription **termination** sequences at the 3'-end of luciferase must be removed so that when the pre-RNA, transcribed from the plasmid DNA luciferase. . .

DETD . . . expression, is enhanced by several modifications to the pVGELVIS-SINBV-luc vector. These modifications include alternate RNA polymerase If promoters and transcription **termination** signals, the addition of intron sequences and ribozyme processing signals in the vector construct, and substitution with a smaller plasmid. . .

DETD The transcription **termination** signals from the SV40 early region or Bovine growth hormone are inserted between the Sac I and Eco RI sites of pBGS 131. The SV40 nts between viral nts 2643 to 2563 containing the early region transcription **termination** sequences are isolated by PCR amplification using the primer pair shown below and the pBR322/SV40 plasmid (ATCC # 45019) as. . .

DETD The Bovine growth hormone transcription **termination** sequences are isolated by PCR amplification using the primer pair shown below and the



DETD In additional modifications to the ELVIS vector, the transcription **termination** sequences are fused directly to the 3'-end Sindbis sequences, resulting in deletion of the polyadenylate tract; or alternatively the antigenomic. . . ribozyme sequence of hepatitis delta virus (HDV) is inserted between the 3'-polyadenylate tract of the ELVIS vector and the transcription **termination** signals.

DETD In the second vector 3'-end configuration, the SV40 or BGH transcription **termination** signals are fused directly to the 3'-end of the ELVIS vector corresponding to Sindbis nt 11,700 and the polyadenylate tract.

DETD . . . a polyadenylate tract are combined with the plasmid constructions containing the HDV ribozyme sequence and the SV40 or BGH transcription **termination** signals. This construction corresponds to the insertion of pKSSINBV and pKSSINBV-luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids. Alternatively,. . . precisely at the viral 3' end corresponding to viral nt 11,700 are linked directly to the SV40 or BGH transcription **termination** signals. This construction corresponds to the insertion of pKSSINBVd and pKSSINBVd1A-luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids.

DETD Using the same overlapping PCR approach, the **CMV** promoter is positioned at the 5' viral end such that transcription initiation results in the addition of a single non-viral nucleotide at the Sindbis 5' end. Amplification of the **CMV** promoter in the first primary PCR reaction is accomplished in a reaction containing the pCDNA3 plasmid and the following primer. . .

DETD Forward primer: pCBgl233F (buffer sequence/Bgl II recognition sequence/**CMV** promoter nts 1-22):

DETD Reverse primer: SNCMV1142R (SIN nts 8-1/**CMV** pro nts 1142-1108):

DETD Forward primer: CMVSIN1F (**CMV** pro nts 11261142/SIN nts 1-20):

DETD Forward primer: pCBgl233F (buffer sequence/Bgl II recognition sequence/**CMV** promoter nts 1-22):

DETD . . . plasmid DNA (FIG. 22A). The data also demonstrate that direct linkage between the Sindbis virus 3'-end and two different transcription **termination**/polyadenylation signals, resulting in deletion of the synthetic A25 tract, decreased the activity of the DNA vector by more than three. . .

DETD . . . of in vitro synthesized transcripts, the cDNA is exchanged into a vector containing an RNA polymerase II promoter and transcription **termination**/polyadenylation sequence, as described in Example 2. For plant applications, such promoter and **termination** sequences are chosen from the appropriate plant systems (e.g., CaMV 35S promoter (Guilley et al., Cell 30:763-773, 1982), and nopaline synthase promoter and transcription **termination** sequence (Sanders et al., Nucleic Acids Res. 15:1543-1558). Vector constructs derived from these infectious genomic cDNA clones is subsequently accomplished. . .

DETD . . . downstream of the disabled junction region in the pKSSINBVd1JR vector, which allows ribosomal scanning in genomic mRNA from non-structural gene **termination** to the heterologous genes. The heterologous proteins are expressed from genomic length mRNA by ribosomal scanning. This extends the life. . .

DETD . . . et al., Gene 108:167-174, 1991). The sequences surrounding the ATG start codon from bases -9 to +1 conform to the **Kozak** consensus sequence for efficient translational initiation (**Kozak**, Cell 44:283-292, 1986). Where possible, the 3' terminal nucleotide corresponding to the carboxy terminal amino acid is changed to T, . . .

DETD . . . gpt, for the current neomycin resistance, or other promoter elements, for example Drosophila metallothionein or hsp 70, for the current **CMV**, MuLV, and SV40 promoters, may be readily accomplished given the disclosure provided herein.

DETD . . . map, respectively, to the authentic AUG translational start and UGA translational stop sites, including the surrounding nucleotides corresponding to the **Kozak** consensus sequence for efficient translational initiation at Sindbis nt 7638. The forward primer is complementary to Sindbis nts 7638-7661, and. . .

DETD . . . structural protein gene MnRNA, the alphavirus structural protein gene sequences, the 3'-end alphavirus sequences required for replication, and a transcription **termination**/polyadenylation sequence. Because of an upstream open-reading frame which ends in translation **termination** codons prior to the AUG start site of the structural protein genes, expression of the alphavirus structural proteins can occur. . .

DETD In addition to their respective complementarities, primer SIN276-SPE contains a flanking UAA translation **termination** codon and SpeI recognition sequence at its 5' end. PCR amplification of the fragment containing Sindbis 5'-end sequences fused to. . .

DETD Modifications of the **CMV** promoter/antisense-Sindbis structural protein vector also can be constructed using other viral, cellular, or insect-based promoters. Using common molecular biology techniques know in the art, the **CMV** promoter can be switched out of the Invitrogen pCDNA3 vector and replaced by promoters such as those listed previously. Other. . . use of longer or shorter HDV or other catalytic ribozyme

transcription **termination** signal for the catalytic ribozyme sequence, or the antisense expression of structural protein gene cassettes using any downstream sequence recognized. . . .

DETD . . . vector and the Astrovirus expression cassette, between the Astrovirus junction region and structural gene ATG, and between the structural gene **termination** codon and the Astrovirus 3' end. In order to generate wild type virus, this dual recombination event would have to. . . .

DETD . . . glycoproteins E1 and/or E2 from other alphaviruses or their variants is performed to alter tissue tropism. For example, Venezuelan equine **encephalitis virus** (VEE) is an alphavirus which exhibits tropism for cells of lymphoid origin, unlike its Sindbis virus counterpart. Therefore, Sindbis-derived vector. . . .

DETD . . . is amplified with a primer pair whose 5'-ends map, respectively, to the authentic AUG translational start site, including the surrounding **Kozak** consensus sequence, and UGA translational stop site. The forward primer is complementary to VEE nucleotides 7553-7579, and the reverse primer. . . .

DETD . . . Madison, Wis.) by digestion of pcDNA3 with Apa I and EcoRV followed by GENECLAN.TM. purification. The resulting construct, containing a **CMV** promoter which transcribes an antisense reporter cassette RNA of the configuration Sindbis 3'-end sequence/LacZ gene/junction region/Sindbis 5'-end sequence/HDV ribozyme, is. . . .

DETD . . . vector. The re-ligated construct contains an antisense LacZ gene and a 3' Sindbis replicase protein recognition sequence downstream from a **CMV** promoter. The resulting construct is known as pcDNAaLacZ-3'Sin. The construct is transfected into BHK cells and utilized as described previously.

DETD . . . assays; (d) map immune response epitopes; and (e) elicit and measure CTL responses to other non-HIV antigens such as mouse **CMV** (MCMV).

DETD Subgenomic mRNA is synthesized in these vectors, which serves as a translational template for the Ad E3 and **CMV** H301 genes. Thus, in these constructions, functional HPV 16 antisense and hairpin ribozyme palliatives will be present on the levels. . . .

DETD . . . the E6/E7 or LI proteins. In these vectors, it would not be desired to include the immunoregulatory Ad E3 or **CMV** H301 genes.

DETD . . . among the group described in Example 5, and the therapeutic palliative. Ordered insertion of the hairpin ribozyme, Ad E3 or **CMV** H301, IRES, and heterologous gene of interest components along the multiple cloning sequence located in the vector between the vector. . . .

DETD The coding region and transcriptional **termination** signals of HSV-1 thymidine kinase gene (HSV-TK) are isolated as a 1.8 kb Bgl II/Pvu II fragment from plasmid 322TK. . . .

. . . selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, HBV, HIV, HSV, FeLV, FIV, HTLV-1, HTLV-2, and **CMV**.

L8 ANSWER 4 OF 11 USPATFULL on STN

2000:7187 Eukaryotic layered vector initiation systems.

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 US 6015686 20000118

**APPLICATION: US 1995-404796 19950315 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A eukaryotic layered vector initiation system comprising a eukaryotic promoter 5' of viral cDNA which initiates within a cell the 5' to 3' synthesis of RNA from cDNA, wherein said RNA comprises a vector construct which autonomously amplifies in a cell, said vector construct expressing a heterologous nucleic acid sequence.

2. The eukaryotic layered vector initiation system according to claim 1, wherein said 5' promoter is a DNA promoter of RNA synthesis.

3. The eukaryotic layered vector initiation system according to claim 1, wherein said vector construct which autonomously amplifies comprises a sequence which initiates transcription of alphavirus RNA following said eukaryotic promoter 5' of viral cDNA, a nucleic acid sequence which encodes alphavirus nonstructural proteins, an alphavirus RNA polymerase recognition sequence and a 3' polyadenylate tract.

4. The eukaryotic layered vector initiation system according to claim 1, further comprising a transcription **termination** sequence.

5. The eukaryotic layered vector initiation system according to claim 1, wherein said vector construct which autonomously amplifies is derived from a virus selected from the group consisting of poliovirus,

Astrovirus.

6. The eukaryotic layered vector initiation system according to claim 1, wherein said vector construct which autonomously amplifies is derived from a virus selected from the group consisting of tobamoviruses, potyviruses and bromoviruses.

7. The eukaryotic layered vector initiation system according to claim 1, wherein said promoter is selected from the group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40 promoter, CaMV 35S promoter, nopaline synthetase promoter, and the **CMV** promoter.

8. The eukaryotic layered vector initiation system according to claim 1, wherein said heterologous sequence is a sequence encoding a protein selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15,  $\alpha$ -IFN,  $\beta$ -IFN,  $\gamma$ -IFN, G-CSF, and GM-CSF.

9. The eukaryotic layered vector initiation system according to claim 1, wherein said heterologous sequence is obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hantavirus, HTLV I, HTLV II, and **CMV**.

10. The eukaryotic layered vector initiation system according to claim 1, wherein said heterologous sequence is an antisense sequence, a non-coding sense sequence or ribozyme sequence.

11. The eukaryotic layered vector initiation system according to claim 10, wherein said antisense sequence or non-coding sense sequence is selected from the group consisting of sequences which are complementary to influenza virus, respiratory syncytial virus, HPV, HBV, HCV, EBV, HIV, HSV, and **CMV** sequences.

12. A host cell containing a eukaryotic layered vector initiation system according to any one of claims 1 to 11.

13. The host cell according to claim 12 wherein said cell is a eukaryotic cell.

14. The host cell according to claim 12 wherein the eukaryotic layered vector initiation system is stably integrated.

15. The host cell according to claim 12 wherein said cell is a mammalian cell.

16. A method for producing one or more recombinant proteins, comprising growing, under suitable nutrient conditions, eukaryotic host cells transformed or transfected with a eukaryotic layered vector initiation system according to claim 1 in a manner allowing expression of said heterologous sequence.

17. The method according to claim 16 wherein said recombinant protein is selected from the group consisting of an interleukin, an interferon, insulin, hemoglobin, EP G-CSF, GM-CSF, M-CSF, SCF, MGDF, the flt3 ligand, BDNF, NT-3, CNTF, NGF, PDGF, FGF, EGF, KGF, factor VIII, factor IX, t-PA, streptokinase, human growth hormone, ICAM-1, and ELAM.

18. A method for delivering a heterologous nucleic acid sequence to an animal, comprising administering to said animal a eukaryotic layered vector initiation system according to claim 1.

19. A method for producing a recombinant protein, comprising administering to a tissue of an animal a eukaryotic layered vector initiation system according to claim 1, wherein the eukaryotic layered vector initiation system comprises a selected heterologous nucleotide sequence which is expressed upon introduction into the tissue of said animal.

20. A method for producing packaged vector particles, comprising introducing a eukaryotic layered vector initiation system according to claim 1 into a packaging cell line.

<b>AI</b>	<b>US 1995-404796</b>	<b>19950315 (8)</b>
SUMM	. . . transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription <b>termination</b> .	
SUMM	. . . transcription of the subgenomic fragment is reduced, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription <b>termination</b> .	
SUMM	. . . transcription of the subgenomic fragment is reduced, an	

controls transcription **termination**.

SUMM . . . cell, the vector construct being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**.

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SUMM . . . from cDNA is selected from the group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40 promoter, and the **CMV** promoter. Within further embodiments, the eukaryotic layered vector initiation systems further comprise a polyadenylation sequence.

SUMM . . . group consisting of influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and **CMV**. Within one preferred embodiment, the heterologous sequence obtained from HPV encodes a protein selected from the group consisting of E5, . . .

SUMM . . . are complementary to influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and **CMV** sequences.

SUMM . . . 5C distal, SV40, heat shock protein 65, heat shock protein 70, Py, RSV, BK, JC, MuLV, MMTV, alphavirus junction region, **CMV** and VA1RNA.

DETD . . . reduce viral transcription of the subgenomic fragment, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**. The vector may also include nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, . . .

DETD . . . sequence encoding one or more proteins, said proteins preferably comprising alphavirus structural protein(s). Optionally, the expression cassette may include transcription **termination**, splice recognition, and polyadenylation addition sites. Preferred promoters include the **CMV**, MMTV, MoMLV, and adenovirus VA1RNA promoters. In addition, the expression cassette may contain selectable markers such as Neo, SV2 Neo, . . .

DETD . . . promoters, such as, for example, the  $\beta$ -galactosidase promoter, trpE promoter, lacZ promoter, T7 promoter, T3 promoter, SP6 promoter, SV40 promoter, **CMV** promoter, and MoMLV LTR.

DETD 4. The **CMV** H301 Gene

DETD . . . the human immunoglobulin heavy-chain binding protein, and a synthetic sequence of at least 15 bps corresponding in part to the **Kozak** consensus sequence for efficient translational initiation. Although not described in detail here, these signals which affect translation initiation can also. . .

DETD As noted above, the alphavirus cDNA vector construct also includes a 3' sequence which controls transcription **termination**. A representative example of such a sequence is set forth in more detail below in Examples 2 and 3.

DETD . . . vector may be selected to hybridize to any pathogen-specific RNA, for instance target cells infected by pathogens such as HIV, **CMV**, HBV, HPV and HSV.

DETD . . . that any truncation leave intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational **termination** codons may be introduced downstream of the immunogenic region. Insertion of **termination** codons will prematurely terminate protein expression, thus preventing expression of the transforming portion of the protein.

DETD . . . "HCV", respectively), Human Papiloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, HTLV I, HTLV II and Cytomegalovirus ("**CMV**"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which. . .

DETD . . . cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**. Briefly, such eukaryotic layered vector initiation systems provide a two-stage or "layered" mechanism which controls expression of heterologous nucleotide sequences. . . . promoter which is capable of initiating the 5' synthesis of RNA from cDNA (e.g., a 5' promoter), a 3' transcription **termination** site, as well as one or more splice sites and/or a polyadenylation site, if desired. Representative promoters suitable for use. . . Mouse polyoma virus promoter ("Py"), rous sarcoma virus ("RSV"), BK virus and JC virus promoters, MMTV promoter, alphavirus junction region, **CMV** promoter, Adenovirus VA1RNA, rRNA promoter, tRNA methionine promoter, CaMV 35S promoter, nopaline synthetase promoter, and the lac promoter. The second. . .

DETD . . . example, vector systems derived from viruses of the following families: Picornaviridae (e.g., poliovirus, rhinovirus, coxsackieviruses), Caliciviridae, Togaviridae (e.g. alphavirus, rubella), **Flaviviridae** (e.g., yellow fever), Coronaviridae (e.g., HCV, TGEV, IBV, MHV, BCV), Bunyaviridae, Arenaviridae, Retroviridae (e.g., RSV, MoMLV, HIV, HTLV), hepatitis delta. . .

DETD . . . transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**. Within various embodiments, the

the subgenomic fragment is merely. . .

DETD . . . auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV HTLV I, HTLV II, **CMV**, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease.

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DETD . . . are observed among the eight alphaviruses sequenced (Semliki Forest virus, Middleburg virus, Ross River virus, O'Nyong Nyong virus, Eastern Equine **Encephalitis virus**, Western Equine **Encephalitis virus**, and Venezuelan Equine **Encephalitis virus**) are given below:

DETD In order to insert the luciferase gene into the pVGELVIS-SINBV vector, the SV40 intron and transcription **termination** sequences at the 3'-end of luciferase must be removed so that when the pre-RNA, transcribed from the plasmid DNA luciferase. . .

DETD . . . expression, is enhanced by several modifications to the pVGELVIS-SINBV-luc vector. These modifications include alternate RNA polymerase II promoters and transcription **termination** signals, the addition of intron sequences and ribozyme processing signals in the vector construct, and substitution with a smaller plasmid. . .

DETD The transcription **termination** signals from the SV40 early region or Bovine growth hormone are inserted between the Sac I and Eco RI sites of pBGS131. The SV40 nts between viral nts 2643 to 2563 containing the early region transcription **termination** sequences are isolated by PCR amplification using the primer pair shown below and the pBR322/SV40 plasmid (ATCC # 45019) as. . .

DETD The Bovine growth hormone transcription **termination** sequences are isolated by PCR amplification using the primer pair shown below and the pCDNA3 plasmid (Invitrogen) as template.

DETD In additional modifications to the ELVIS vector, the transcription **termination** sequences are fused directly to the 3'-end Sindbis sequences, resulting in deletion of the polyadenylate tract; or alternatively the antigenomic. . . ribozyme sequence of hepatitis delta virus (HDV) is inserted between the 3'-polyadenylate tract of the ELVIS vector and the transcription **termination** signals.

DETD In the second vector 3'-end configuration, the SV40 or BGH transcription **termination** signals are fused directly to the 3'-end of the ELVIS vector corresponding to Sindbis nt 11,700 and the polyadenylate tract.

DETD . . . a polyadenylate tract are combined with the plasmid constructions containing the HDV ribozyme sequence and the SV40 or BGH transcription **termination** signals. This construction corresponds to the insertion of pKSSINBV and pKSSINBV-luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids. Alternatively,. . . precisely at the viral 3' end corresponding to viral nt 11,700 are linked directly to the SV40 or BGH transcription **termination** signals. This construction corresponds to the insertion of pKSSINBVd1A and pKSSINBVd1A-luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids.

DETD Using the same overlapping PCR approach, the **CMV** promoter is positioned at the 5' viral end such that transcription initiation results in the addition of a single non-viral nucleotide at the Sindbis 5' end. Amplification of the **CMV** promoter in the first primary PCR reaction is accomplished in a reaction containing the pCDNA3 plasmid and the following primer. . .

DETD Forward primer: pCBgl233F buffer sequence/Bgl II recognition sequence/**CMV** promoter nts 1-22)

DETD Reverse primer: SNCMV1142R (SIN nts 8-1/**CMV** pro nts 1142-1108)

DETD Forward primer: CMVSIN1F (**CMV** pro nts 1124-1142/SIN nts 1-20)

DETD Forward primer: pCBgl233F (buffer sequence/Bgl II recognition sequence/**CMV** promoter nts 1-22)

DETD . . . plasmid DNA (FIG. 22A). The data also demonstrate that direct linkage between the Sindbis virus 3'-end and two different transcription **termination**/polyadenylation signals, resulting in deletion of the synthetic A<sub>25</sub> tract, decreased the activity of the DNA vector by more than three. . .

DETD . . . of in vitro synthesized transcripts, the cDNA is exchanged into a vector containing an RNA polymerase II promoter and transcription **termination**/polyadenylation sequence, as described in Example 2. For plant applications, such promoter and **termination** sequences are chosen from the appropriate plant systems (e.g., CaMV 35S promoter (Guilley et

transcription **termination** sequence (Sanders et al., Nucleic Acids Res. 15:1543-1558). Vector constructs derived from these infectious genomic cDNA clones is subsequently accomplished.

DETD . . . downstream of the disabled junction region in the pKSSINBVd1JR vector, which allows ribosomal scanning in genomic mRNA from non-structural gene **termination** to the heterologous genes. The heterologous proteins are expressed from genomic length mRNA by ribosomal scanning. This extends the life.

DETD . . . et al., Gene 108:167-174, 1991). The sequences surrounding the ATG start codon from bases -9 to +1 conform to the **Kozak** consensus sequence for efficient translational initiation (**Kozak**, Cell 44:283-292, 1986). Where possible, the 3' terminal nucleotide corresponding to the carboxy terminal amino acid is changed to T.

DETD . . . gpt, for the current neomycin resistance, or other promoter elements, for example Drosophila metallothionein or hsp 70, for the current **CMV**, MuLV, and SV40 promoters, may be readily accomplished given the disclosure provided herein.

DETD . . . map, respectively, to the authentic AUG translational start and UGA translational stop sites, including the surrounding nucleotides corresponding to the **Kozak** consensus sequence for efficient translational initiation at Sindbis nt 7638. The forward primer is complementary to Sindbis nts 7638-7661, and.

DETD . . . structural protein gene mRNA, the alphavirus structural protein gene sequences, the 3'-end alphavirus sequences required for replication, and a transcription **termination**/polyadenylation sequence. Because of an upstream open-reading frame which ends in translation **termination** codons prior to the AUG start site of the structural protein genes, expression of the alphavirus structural proteins can occur.

DETD In addition to their respective complementarities, primer SIN276-SPE contains a flanking UAA translation **termination** codon and SpeI recognition sequence at its 5' end. PCR amplification of the fragment containing Sindbis 5'-end sequences fused to.

DETD Modifications of the **CMV** promoter/antisense-Sindbis structural protein vector also can be constructed using other viral, cellular, or insect-based promoters. Using common molecular biology techniques known in the art, the **CMV** promoter can be switched out of the Invitrogen pcDNA3 vector and replaced by promoters such as those listed previously. Other . . . use of longer or shorter HDV or other catalytic ribozyme sequences for transcript processing, the substitution of a precise transcription **termination** signal for the catalytic ribozyme sequence, or the antisense expression of structural protein gene cassettes using any downstream sequence recognized.

DETD . . . vector and the Astrovirus expression cassette, between the Astrovirus junction region and structural gene ATG, and between the structural gene **termination** codon and the Astrovirus 3' end. In order to generate wild type virus, this dual recombination event would have to.

DETD . . . glycoproteins E1 and/or E2 from other alphaviruses or their variants is performed to alter tissue tropism. For example, Venezuelan equine **encephalitis virus** (VEE) is an alphavirus which exhibits tropism for cells of lymphoid origin, unlike its Sindbis virus counterpart. Therefore, Sindbis-derived vector.

DETD . . . is amplified with a primer pair whose 5'-ends map, respectively, to the authentic AUG translational start site, including the surrounding **Kozak** consensus sequence, and UGA translational stop site. The forward primer is complementary to VEE nucleotides 7553-7579, and the reverse primer.

DETD . . . Madison, Wis.) by digestion of pcDNA3 with Apa I and EcoRV followed by GENECLAN.TM. purification. The resulting construct, containing a **CMV** promoter which transcribes an antisense reporter cassette RNA of the configuration Sindbis 3'-end sequence/LacZ gene/junction region/Sindbis 5'-end sequence/HDV ribozyme, is.

DETD . . . vector. The re-ligated construct contains an antisense LacZ gene and a 3' Sindbis replicase protein recognition sequence downstream from a **CMV** promoter. The resulting construct is known as pcDNAaLacZ-3'Sin. The construct is transfected into BHK cells and utilized as described previously.

DETD . . . assays; (d) map immune response epitopes; and (e) elicit and measure CTL responses to other non-HIV antigens such as mouse **CMV** (MCMV).

DETD Subgenomic mRNA is synthesized in these vectors, which serves as a translational template for the Ad E3 and **CMV** H301 genes. Thus, in these constructions, functional HPV 16 antisense and hairpin ribozyme palliatives will be present on the levels.

DETD . . . the E6/E7 or L1 proteins. In these vectors, it would not be desired to include the immunoregulatory Ad E3 or **CMV** H301 genes.

DETD . . . among the group described in Example 5, and the therapeutic palliative. Ordered insertion of the hairpin ribozyme, Ad E3 or **CMV** H301, IRES, and heterologous gene of interest components along the multiple cloning sequence located in the vector between the vector.

DETD The coding region and transcriptional **termination** signals of HSV-1

fragment from plasmid 322TK.

4. The eukaryotic layered vector initiation system according to claim 1, further comprising a transcription **termination** sequence.

group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40 promoter, CaMV 35S promoter, nopaline synthetase promoter, and the **CMV** promoter.

of influenza virus, respiratory syncytial virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hantavirus, HTLV I, HTLV II, and **CMV**.

group consisting of sequences which are complementary to influenza virus, respiratory syncytial virus, HPV, HBV, HCV, EBV, HIV, HSV, and **CMV** sequences.

L8 ANSWER 5 OF 11 USPTAFULL on STN

1999:141912 Compositions and methods for delivery of genetic material.

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US 5981505 19991109

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**APPLICATION: US 1997-979385 19971126 (8)**

**WO 1994-US899 19940126 19950828 PCT 371 date 19950828 PCT 102(e) date**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A pharmaceutical composition comprising: a) a polynucleotide function enhancer; and b) a DNA molecule that comprises a DNA sequence that encodes an antigen; wherein said polynucleotide function enhancer is selected from the group consisting of bupivacaine and tetracaine and said DNA sequence operatively linked to regulatory sequences which control the expression of said DNA sequence.

2. The pharmaceutical composition of claim 1 wherein said composition comprises bupivacaine.

3. The pharmaceutical composition of claim 1 wherein said composition comprises tetracaine.

4. The pharmaceutical composition of claim 1 wherein said DNA molecule is a plasmid.

5. The pharmaceutical composition of claim 1 wherein said DNA sequence encodes a variable region of a T cell receptor.

6. The pharmaceutical composition of claim 1 wherein said DNA sequence encodes a pathogen antigen.

7. The pharmaceutical composition of claim 6 wherein said DNA sequence encodes an antigen from an intracellular pathogen.

8. The pharmaceutical composition of claim 7 wherein said antigen is a viral antigen.

9. The pharmaceutical composition of claim 7 wherein said pathogen is a virus selected from the group consisting of: human immunodeficiency virus, HIV; human T cell leukemia virus, HTLV; influenza virus; hepatitis A virus; hepatitis B virus; hepatitis C virus; human papilloma virus, HPV; Herpes simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus, **CMV**; Epstein-Barr virus, EBR; rhinovirus; and, coronavirus.

10. The pharmaceutical composition of claim 9 wherein said pathogen is Herpes simplex 2 virus, HSV2.

11. The pharmaceutical composition of claim 9 wherein said pathogen is Hepatitis B virus, HBV.

12. The pharmaceutical composition of claim 9 wherein said pathogen is human T cell leukemia virus, HTLV.

13. The pharmaceutical composition of claim 9 wherein said pathogen is a human immunodeficiency virus.

14. The pharmaceutical composition of claim 1 wherein said DNA sequence encodes a hyperproliferative disease associated protein.

hyperproliferative disease is cancer.

16. The pharmaceutical composition of claim 14 wherein said hyperproliferative disease is a lymphoma.

17. The pharmaceutical composition of claim 14 wherein said hyperproliferative disease is a melanoma.

18. A method of immunizing an individual comprising the steps of: injecting into tissue of said individual at a site on said individual's body, a DNA molecule and a polynucleotide function enhancer, said DNA molecule comprising a DNA sequence that encodes an antigen, said DNA sequence operatively linked to regulatory sequences which control the expression of said DNA sequence, said polynucleotide function enhancer selected from the group consisting of bupivacaine and tetracaine; wherein said DNA molecule is taken up by cells in said tissue, said DNA sequence is expressed in said cells and an immune response is generated against said antigen.

19. The method of claim 18 wherein said tissue includes skin and skeletal muscle.

20. The method of claim 18 wherein said tissue is skin.

21. The method of claim 18 wherein said tissue is muscle.

22. The method of claim 18 wherein said tissue is skeletal muscle.

23. The method of claim 18 wherein said polynucleotide function enhancer is bupivacaine.

24. The method of claim 18 wherein said polynucleotide function enhancer is tetracaine.

25. The method of claim 18 wherein said DNA molecule is a plasmid.

26. The method of claim 18 wherein said immune response generated against said antigen is an immune response against a pathogen antigen.

27. The method of claim 26 wherein said pathogen is an intracellular pathogen.

28. The method of claim 27 wherein said intracellular pathogen is a virus.

29. The method of claim 26 wherein said pathogen is a virus selected from the group consisting of: human immunodeficiency virus, HIV; human T cell leukemia virus, HTLV; influenza virus; hepatitis A virus; hepatitis B virus; hepatitis C virus; human papilloma virus, HPV; Herpes simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus, CMV; Epstein-Barr virus, EBR; rhinovirus; and, coronavirus.

30. The method of claim 18 wherein said immune response generated against said antigen provides a protective immune response against a pathogen and said individual is immunized against said pathogen.

31. The method of claim 30 wherein said tissue is skin.

32. The method of claim 30 wherein said tissue is skeletal muscle.

33. The method of claim 30 wherein said polynucleotide function enhancer is bupivacaine.

34. The method of claim 30 wherein said polynucleotide function enhancer is tetracaine.

35. The method of claim 30 wherein said DNA molecule is a plasmid.

36. The method of claim 30 wherein said antigen is a pathogen antigen.

37. The method of claim 30 wherein said pathogen is an intracellular pathogen.

38. The method of claim 37 wherein said intracellular pathogen is a virus.

39. The method of claim 38 wherein said virus is selected from the group consisting of: human immunodeficiency virus, HIV; human T cell leukemia virus, HTLV; influenza virus; hepatitis A virus; hepatitis B virus; hepatitis C virus; human papilloma virus, HPV; Herpes simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus, CMV; Epstein-Barr virus, EBR; rhinovirus; and, coronavirus.



40. The method of claim 39 wherein said virus is Herpes simplex 2 virus, HSV2.
41. The method of claim 39 wherein said virus is Hepatitis B virus, HBV.
42. The method of claim 39 wherein said virus is human T cell leukemia virus, HTLV.
43. The method of claim 18 wherein said immune response generated against said antigen provides a therapeutic immune response against a pathogen in an individual who is infected with said pathogen.
44. The method of claim 43 wherein said tissue is skin.
45. The method of claim 43 wherein said tissue is skeletal muscle.
46. The method of claim 43 wherein said polynucleotide function enhancer is bupivacaine.
47. The method of claim 43 wherein said polynucleotide function enhancer is tetracaine.
48. The method of claim 43 wherein said DNA molecule is a plasmid.
49. The method of claim 43 wherein said pathogen is an intracellular pathogen.
50. The method of claim 43 wherein said pathogen is a virus.
51. The method of claim 18 wherein said immune response generated against said antigen is an immune response against a hyperproliferative disease-associated protein.
52. The method of claim 51 wherein said tissue is skin.
53. The method of claim 51 wherein said tissue is skeletal muscle.
54. The method of claim 51 wherein said polynucleotide function enhancer is bupivacaine.
55. The method of claim 51 wherein said polynucleotide function enhancer is tetracaine.
56. The method of claim 51 wherein said DNA molecule is a plasmid.
57. The method of claim 51 wherein said hyperproliferative disease-associated protein is selected from the group consisting of: protein products of oncogenes myb, myc, fyn, ras, src, neu and trk; protein products of translocation gene bcr/abl; P53; variable regions of antibodies made by B cell lymphomas; and variable regions of T cell receptors of T cell lymphomas.
58. The method of claim 51 wherein said immune response generated against said antigen is a therapeutically effective immune response against a hyperproliferative disease-associated protein in an individual who has a hyperproliferative disease.
59. The method of claim 58 wherein said hyperproliferative disease is cancer.
60. The method of claim 58 wherein said hyperproliferative disease is a melanoma.
61. The method of claim 58 wherein said hyperproliferative disease is a lymphoma.
62. The method of claim 18 wherein said immune response generated against said antigen is an immune response against an autoimmune disease-associated protein.
63. The method of claim 62 wherein said tissue is skin.
64. The method of claim 62 wherein said tissue is skeletal muscle.
65. The method of claim 62 wherein said polynucleotide function enhancer is bupivacaine.
66. The method of claim 62 wherein said polynucleotide function enhancer is tetracaine.
67. The method of claim 62 wherein said DNA molecule is a plasmid.

associated-protein is selected from the group consisting of: variable regions of antibodies involved in B cell mediated autoimmune disease; and variable regions of T cell receptors involved in T cell mediated autoimmune disease.

69. A method of introducing DNA molecules into cells of an individual comprising the step of: injecting into tissue of said individual at a site on said individual's body, DNA molecules and a polynucleotide function enhancer selected from the group consisting of bupivacaine and tetracaine, wherein said DNA molecules are taken up by cells in said tissue.

70. The method of claim 69 wherein said DNA molecule comprises a DNA sequence that encodes an protein, said DNA sequence operatively linked to regulatory sequences which control the expression of said DNA sequence.

71. The method of claim 69 wherein said tissue is skin.

72. The method of claim 69 wherein said tissue is skeletal muscle.

73. The method of claim 69 wherein said polynucleotide function enhancer is bupivacaine.

74. The method of claim 69 wherein said polynucleotide function enhancer is tetracaine.

75. The method of claim 69 wherein said DNA molecule is a plasmid.

<b>AI</b>	<b>US 1997-979385</b>	<b>19971126 (8)</b>
	<b>WO 1994-US899</b>	<b>19940126</b>
		19950828 PCT 371 date
		19950828 PCT 102(e) date
DETD	. . . individual. Regulatory elements for DNA expression include a promoter and a polyadenylation signal. In addition, other elements, such as a <b>Kozak</b> region, may also be included in the genetic construct.	
DETD	. . . the DNA or RNA molecule that comprises a nucleotide sequence which encodes the desired protein and which includes initiation and <b>termination</b> signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of. . .	
DETD	. . . is necessary that these elements are functional in the individual to whom the gene construct is administered. The initiation and <b>termination</b> codons must be in frame with the coding sequence.	
DETD	. . . Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus ( <b>CMV</b> ) such as the <b>CMV</b> immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as. . .	
DETD	. . . but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from <b>CMV</b> , RSV and EBV.	
DETD	. . . and a polyadenylation signal. The promoter may be selected from the group consisting of: HIV LTR, human Actin, human Myosin, <b>CMV</b> , RSV, Moloney, MMTV, human Hemoglobin, human muscle creatine and EBV. The enhancer may be selected from the group consisting of: human Actin, human Myosin, <b>CMV</b> , RSV, human Hemoglobin, human muscle creatine and EBV. The polyadenylation signal may be selected from the group consisting of: LTR. . .	
DETD	A region from just upstream of the unique PflMI site to just after the vif <b>termination</b> codon was amplified via PCR using primers that introduced a non-conservative amino acid change (glu→val) at amino acid 22 of. . .	
DETD	. . . HXB2 was amplified via PCR and cloned into the expression vector pCND4/neo (Invitrogen). This plasmid drives envelope production through the <b>CMV</b> promoter.	
DETD	. . . expression is MMTV LTR. The promoter may be deleted and replaced with Actin promoter, myosin promoter, HIV LTR promoter and <b>CMV</b> promoter.	
DETD	. . . Actin no no yes	
	RA-4 Actin CME yes yes	
	RA-5 Actin CME yes no	
	RA-6 Actin CME no yes	
	RA-7 <b>CMV</b> no yes yes	
	RA-8 <b>CMV</b> no yes no	
	RA-9 <b>CMV</b> no no yes	
	RA-10 <b>CMV</b> CME yes yes	
	RA-11 <b>CMV</b> CME yes no	
	RA-12 <b>CMV</b> CME no yes	
	RA-13 MMTV no yes yes	
	RA-14 MMTV no yes no	
	RA-15 MMTV no no yes	

DETD . . . The HIV 5'LTR promoter can be deleted and replaced with Moloney virus promoter, MMTV LTR, Actin promoter, myosin promoter and **CMV** promoter.

DETD . . . Moloney HIV 3' LTR yes

LA-2 Moloney SV40 yes

LA-3 Moloney HIV 3' LTR no

LA-4 Moloney SV40 no

LA-5 **CMV** HIV 3' LTR yes

LA-6 CNV SV40 yes

LA-7 **CMV** HIV 3' LTR no

LA-8 **CMV** SV40 no

LA-9 MMTV HIV 3' LTR yes

LA-10 MMTV SV40 yes

LA-11 MMTV HIV 3' LTR no

LA-12 MMTV. . .

DETD . . . the thymidine kinase promoter and polyadenylation site. The HIV env coding region is placed under the regulatory control of the **CMV** promoter and SV40 polyadenylation site. The HIV env coding region was obtained as a 2.3 kb PCR fragment from HIV/3B, . . .

DETD . . . the thymidine kinase promoter and polyadenylation site. The HIV gag/pol coding region is placed under the regulatory control of the **CMV** promoter and SV40 polyadenylation site. The HIV gag/pol coding region was obtained from HIV MN, Genebank sequence MI7449, and includes. . .

DETD . . . the thymidine kinase promoter and polyadenylation site. The HIV gag/pol coding region is placed under the regulatory control of the **CMV** promoter and SV40 polyadenylation site. The HIV gag/pol coding region was obtained from HIV MN, Genebank sequence MI7449, and includes. . .

DETD Several safety features are included in pGAGPOL.rev. These include use of the **CMV** promoter and a non-retroviral poly(A) site. Furthermore, deletion of the  $\psi$  sequence limits the ability to package viral RNA. In. . .

DETD Several safety features are included in PGAGPOL.rev. These include use of the **CMV** promoter and a non-retroviral poly(A) site. Furthermore, tat has been deleted and a 50% deletion of nef yields an "inactive". . .

DETD Step 4a. Digest with BamHI and ligate with the **CMV** promoter obtained by PCR of pCEP4 (Invitrogen, San Diego Calif.) with primers SEQ ID NO:25 and SEQ ID NO:26.

DETD . . . be any promoter functional in a human cell. It is preferred that the promoter is an SV40 promoter or a **CMV** promoter, preferably a **CMV** immediate early promoter. The polyadenylation signal may be any polyadenylation signal functional in a human cell. It is preferred that. . . separated by non-coding regions. In order to be properly expressed, a coding region must have an initiation codon and a **termination** codon.

DETD . . . be any promoter functional in a human cell. It is preferred that the promoter is an SV40 promoter or a **CMV** promoter, preferably a immediate early **CMV** promoter. The polyadenylation signal may be any polyadenylation signal functional in a human cell. It is preferred that the polyadenylation. . . separated by non-coding regions. In order to be properly expressed, a coding region must have an initiation codon and a **termination** codon.

DETD . . . identical to or substantially similar to epitopes of HIV proteins. The coding sequence is under the regulatory control of the **CMV** immediate early promoter and the SV40 minor polyadenylation signal.

DETD . . . encodes at least one HIV protein or a fragment thereof. The coding sequence is under the regulatory control of the **CMV** immediate early promoter and the SV40 minor polyadenylation signal. The HIV protein is selected from the group consisting of gag, . . .

DETD . . . identical to or substantially similar to epitopes of HIV proteins. The coding sequence is under the regulatory control of the **CMV** immediate early promoter and the SV40 minor polyadenylation signal. The two expression units are encoded in opposite directions of each. . .

DETD . . . protein or a fragment thereof. Each expression unit comprises a coding sequence that is under the regulatory control of the **CMV** immediate early promoter and the SV40 minor polyadenylation signal. The HIV protein is selected from the group consisting of gag, . . .

DETD Step 9. Cut pCEP4 (Invitrogen) with SalI to release a DNA fragment containing the **CMV** promoter, polylinker, and SV40 poly A site. Purify this fragment and blunt-off with the Klenow fragment of DNA Polymerase I.

DETD . . . fragment obtained in step 9. Isolate plasmid containing the bacterial origin of replication, the Kan<sup>R</sup> gene, the RSV enhancer, the **CMV** promoter, polylinker, and the SV40 poly A site.

DETD . . . coding region of rev containing both the AvaII site and the nucleotide encoding amino acid 81. A stop codon causing **termination** of Nef at amino acid position 63 and the 3' coding cloning site, MluI, will be introduced by the 3'. . .

DETD . . . include Senilis viruses,

Equine encephalitis.  
 Reovirus: (Medical) Rubella virus.  
 Flariviridae Family  
 Examples include: (Medical) **dengue**,  
 yellow fever, Japanese encephalitis, St.  
 Louis encephalitis and tick borne  
 encephalitis viruses.

Hepatitis C Virus: (Medical) these viruses are not placed in  
 a family yet but are believed to be either a togavirus or a  
**flavivirus**. Most similarity is with togavirus family.

Coronavirus Family:  
 (Medical and Veterinary)  
 Infectious bronchitis virus (poultry)  
 Porcine transmissible gastroenteric virus  
 (pig)  
 Porcine. . .

- . . . B virus; hepatitis C virus; human papilloma virus, HPV; Herpes  
 simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus,  
**CMV**; Epstein-Barr virus, EBR; rhinovirus; and, coronavirus.
- . . . B virus; hepatitis C virus; human papilloma virus, HPV; Herpes  
 simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus,  
**CMV**; Epstein-Barr virus, EBR; rhinovirus; and, coronavirus.
- . . . B virus; hepatitis C virus; human papilloma virus, HPV; Herpes  
 simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus,  
**CMV**; Epstein-Barr virus, EBR; rhinovirus; and, coronavirus.

L8 ANSWER 6 OF 11 USPATFULL on STN

1999:121330 Compositions and methods for delivery of genetic material.  
 Carrano, Richard A., Paoli, PA, United States  
 Wang, Bin, Haidian, China  
 Weiner, David B., Merion, PA, United States  
 Apollon, Inc., Malvern, PA, United States (U.S. corporation)The Trustees Of  
 The University of Pennsylvania, Philadelphia, PA, United States (U.S.  
 corporation)  
 US 5962428 19991005  
 WO 9526718 19951012  
**APPLICATION: US 1996-704701 19960916 (8)**  
**WO 1995-US4071 19950330 19960916 PCT 371 date 19960916 PCT 102(e) date**  
 DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A method of generating an immune response in an individual against an  
 antigen comprising administering in vivo to muscle or skin of said  
 individual's body a genetic vaccine facilitator selected from the group  
 consisting of: anionic lipids; saponins; lectins; estrogenic compounds;  
 hydroxylated lower alkyls; dimethyl sulfoxide; and urea; and, a DNA  
 molecule that is dissociated from an infectious agent and comprises a  
 DNA sequence that encodes said antigen, said DNA sequence operatively  
 linked to regulatory sequences which control the expression of said DNA  
 sequence; wherein said DNA molecule is taken up by cells, said DNA  
 sequence is expressed in said cells and an immune response is generated  
 against said antigen.
2. The method of claim 1 wherein said genetic vaccine facilitator is an  
 anionic lipid selected from the group consisting of: salts of lauric and  
 oleic acids, lauric and oleic acids, acid esters of lauryl and cetyl  
 alcohol, and sulfonates.
3. The method of claim 1 wherein said genetic vaccine facilitator is an  
 anionic lipid selected from the group consisting of: sodium lauryl  
 sulfate and oleic acid.
4. The method of claim 1 wherein said genetic vaccine facilitator is a  
 saponin selected from the group consisting of: saponarin,  
 sarmentocymarin and sapogenins.
5. The method of claim 1 wherein said genetic vaccine facilitator is a  
 saponin selected from the group consisting of: sarmentogenin,  
 sarsasapogenin and sarverogenin.
6. The method of claim 1 wherein said genetic vaccine facilitator is a  
 lectin selected from the group consisting of: concanavalin A, abrin,  
 soybean agglutinin and wheat germ agglutinin.
7. The method of claim 1 wherein said genetic vaccine facilitator is  
 concanavalin A.
8. The method of claim 1 wherein said genetic vaccine facilitator is  
 $\beta$ -estradiol.

9. The method of claim 1 wherein said genetic vaccine facilitator is selected from the group consisting of: ethanol, n-propanol, isopropanol and n-butanol.

10. The method of claim 1 wherein said genetic vaccine facilitator is dimethyl sulfoxide.

11. The method of claim 1 wherein said genetic vaccine facilitator is urea.

12. A method of generating an immune response in an individual against a pathogen comprising administering in vivo to muscle or skin of said individual's body a genetic vaccine facilitator selected from the group consisting of: anionic lipids; saponins; lectins; estrogenic compounds; hydroxylated lower alkyls; dimethyl sulfoxide; and urea; and, a DNA molecule that is dissociated from an infectious agent and comprises a DNA sequence that encodes a protein which comprises at least one epitope that is identical or substantially similar to an epitope of a pathogen antigen, said nucleotide sequence being operably linked to regulatory sequences; wherein said DNA molecule is taken up by cells, said DNA sequence is expressed in said cells and an immune response is generated against said antigen.

13. The method of claim 12 wherein said genetic vaccine facilitator is selected from the group consisting of: sodium lauryl sulfate; oleic acid; saponarin; sarmentocymarin; sapogenins; sarmentogenin; sarsasapogenin; sarverogenin; concanavalin A;  $\beta$ -estradiol; ethanol; dimethyl sulfoxide; and urea.

14. The method of claim 12 wherein said DNA molecule is a plasmid.

15. The method of claim 12 wherein said protein is a pathogen antigen or a fragment thereof which is antigenic.

16. The method of claim 12 wherein said DNA molecule is administered intramuscularly.

17. The method of claim 12 wherein said pathogen is a virus selected from the group consisting of: human immunodeficiency virus, HIV; human T cell leukemia virus, HTLV; influenza virus; hepatitis A virus, HAV; hepatitis B virus, HBV; hepatitis C virus, HCV; human papilloma virus, HPV; Herpes simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus, CMV; Epstein-Barr virus, EBV; rhinovirus; and, coronavirus.

18. The method of claim 12 wherein at least two or more different nucleic acid molecules are administered to different cells of an individual; said different nucleic acid molecules each comprising DNA sequences encoding one or more pathogen antigens of the same pathogen.

19. The method of claim 12 wherein said genetic vaccine facilitator and said DNA molecule are administered simultaneously.

20. A method of generating an immune response in an individual against a disease comprising administering in vivo to muscle or skin of said individual's body a genetic vaccine facilitator selected from the group consisting of: anionic lipids; saponins; lectins; estrogenic compounds; hydroxylated lower alkyls; dimethyl sulfoxide; and urea; and, a DNA molecule that is dissociated from an infectious agent and comprises a DNA sequence that encodes a protein which comprises an epitope identical or substantially similar to an epitope of a protein associated with said disease operatively linked to regulatory sequences; wherein said DNA molecule is taken up by cells, said DNA sequence is expressed in said cells and an immune response is generated against said target protein.

21. The method of claim 20 wherein said genetic vaccine facilitator is selected from the group consisting of: sodium lauryl sulfate; oleic acid; saponarin; sarmentocymarin; sapogenins; sarmentogenin; sarsasapogenin; sarverogenin; concanavalin A;  $\beta$ -estradiol; ethanol; dimethyl sulfoxide; and urea.

22. The method of claim 20 wherein said disease is characterized by hyperproliferating cells.

23. The method of claim 20 wherein said disease is an autoimmune disease.

24. The method of claim 20 wherein said DNA molecule is a plasmid.

25. The method of claim 20 wherein said DNA molecule is administered intramuscularly.

sequence that encodes a protein selected from the group consisting of: protein products of oncogenes myb, myc, fyn, ras, sarc, neu and trk; protein products of translocation gene bcl/abl; p53; EGRF; variable regions of antibodies made by B cell lymphomas; and variable regions of T cell receptors of T cell lymphomas.

27. The method of claim 20 wherein said protein is selected from the group consisting of: variable regions of antibodies involved in B cell mediated autoimmune disease; and variable regions of T cell receptors involved in T cell mediated autoimmune disease.

28. A pharmaceutical composition comprising: i) a DNA molecule that is dissociated from an infectious agent and comprises a DNA sequence which encodes a protein wherein said DNA sequence is operably linked to regulatory sequences required for expression in a mammal and said protein is selected from the group consisting of: proteins which comprise at least one epitope that is identical or substantially similar to an epitope of a pathogen antigen; proteins which comprise an epitope identical or substantially similar to an epitope of a protein associated with hyperproliferating cells; and proteins which comprise an epitope identical or substantially similar to an epitope of a protein associated with an autoimmune disease; and ii) a genetic vaccine facilitator selected from the group consisting of anionic lipids; saponins; lectins; estrogenic compounds; hydroxylated lower alkyls; dimethyl sulfoxide; and urea.

29. The pharmaceutical composition of claim 28 wherein said genetic vaccine facilitator is selected from the group consisting of: sodium lauryl sulfate; oleic acid; saponarin; sarmentocymarin; sapogenins; sarmentogenin; sarsasapogenin; sarverogenin; concanavalin A;  $\beta$ -estradiol; ethanol; dimethyl sulfoxide; and urea.

30. A pharmaceutical kit comprising: i) a container that comprises a DNA molecule that is dissociated from an infectious agent and comprises a DNA sequence which encodes a protein wherein said DNA sequence is operably linked to regulatory sequences required for expression in a mammal and said protein is selected from the group consisting of: proteins which comprise at least one epitope that is identical or substantially similar to an epitope of a pathogen antigen; proteins which comprise an epitope identical or substantially similar to an epitope of a protein associated with hyperproliferating cells; and proteins which comprise an epitope identical or substantially similar to an epitope of a protein associated with an autoimmune disease; and ii) a container that comprises a genetic vaccine facilitator selected from the group consisting of: anionic lipids; saponins; lectins; estrogenic compounds; hydroxylated lower alkyls; dimethyl sulfoxide; and urea.

31. The pharmaceutical kit of claim 30 wherein said genetic vaccine facilitator is selected from the group consisting of: sodium lauryl sulfate; oleic acid; saponarin; sarmentocymarin; sapogenins; sarmentogenin; sarsasapogenin; sarverogenin; concanavalin A;  $\beta$ -estradiol; ethanol; dimethyl sulfoxide; and urea.

32. A method of delivering a protein into cells of an individual in vivo comprising administering to muscle or skin of said individual's body a genetic vaccine facilitator selected from the group consisting of: anionic lipids; saponins; lectins; estrogenic compounds; hydroxylated lower alkyls; dimethyl sulfoxide; and urea; and, a DNA molecule that is dissociated from an infectious agent and comprises a DNA sequence that encodes said protein, said DNA sequence operatively linked to regulatory sequences which control the expression of said DNA sequence; wherein said DNA molecule is taken up by cells, said DNA sequence is expressed in said cells producing said protein in said cells.

33. The method of claim 30 wherein said genetic vaccine facilitator is an anionic lipid selected from the group consisting of: salts of lauric and oleic acids, lauric and oleic acids, acid esters of lauryl and cetyl alcohol, and sulfonates.

34. The method of claim 30 wherein said genetic vaccine facilitator is an anionic lipid selected from the group consisting of: sodium lauryl sulfate and oleic acid.

35. The method of claim 30 wherein said genetic vaccine facilitator is a saponin selected from the group consisting of: saponarin, sarmentocymarin and sapogenins.

36. The method of claim 30 wherein said genetic vaccine facilitator is a saponin selected from the group consisting of: sarmentogenin, sarsasapogenin and sarverogenin.

37. The method of claim 30 wherein said genetic vaccine facilitator is a

soybean agglutinin and wheat germ agglutinin.

38. The method of claim 30 wherein said genetic vaccine facilitator is concanavalin A.

39. The method of claim 30 wherein said genetic vaccine facilitator is  $\beta$ -estradiol.

40. The method of claim 30 wherein said genetic vaccine facilitator is selected from the group consisting of: ethanol, n-propanol, isopropanol and n-butanol.

41. The method of claim 30 wherein said genetic vaccine facilitator is dimethyl sulfoxide.

42. The method of claim 30 wherein said genetic vaccine facilitator is urea.

AI US 1996-704701 19960916 (8)  
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19960916 PCT 371 date  
19960916 PCT 102(e) date

DETD . . . individual. Regulatory elements for DNA expression include a promoter and a polyadenylation signal. In addition, other elements, such as a **Kozak** region, may also be included in the genetic construct.

DETD . . . the DNA or RNA molecule that comprises a nucleotide sequence which encodes the desired protein and which includes initiation and **termination** signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of. . .

DETD . . . is necessary that these elements are functional in the individual to whom the gene construct is administered. The initiation and **termination** codons must be in frame with the coding sequence.

DETD . . . Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (**CMV**) such as the **CMV** immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as. . .

DETD . . . but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from **CMV**, RSV and EBV.

DETD . . . and a polyadenylation signal. The promoter may be selected from the group consisting of: HIV LTR, human Actin, human Myosin, **CMV**, RSV, Moloney, MMTV, human Hemoglobin, human muscle creatine and EBV. The enhancer may be selected from the group consisting of: human Actin, human Myosin, **CMV**, RSV, human Hemoglobin, human muscle creatine and EBV. The polyadenylation signal may be selected from the group consisting of: LTR. . .

DETD A region from just upstream of the unique PflMI site to just after the vif **termination** codon was amplified via PCR using primers that introduced a non-conservative amino acid change (glu $\rightarrow$ val) at amino acid 22 of. . .

DETD . . . HXB2 was amplified via PCR and cloned into the expression vector pCND4/neo (Invitrogen). This plasmid drives envelope production through the **CMV** promoter.

DETD . . . expression is MMTV LTR. The promoter may be deleted and replaced with Actin promoter, myosin promoter, HIV LTR promoter and **CMV** promoter.

DETD . . . Actin no yes no

RA-3 Actin no no yes

RA-4 Actin CME yes yes

RA-5 Actin CME yes no

RA-6 Actin CME no yes

RA-7 **CMV** no yes yes

RA-8 **CMV** no yes no

RA-9 **CMV** no no yes

RA-10 **CMV** CME yes yes

RA-11 **CMV** CME yes no

RA-12 **CMV** CME no yes

RA-13 MMTV no yes yes

RA-14 MMTV no yes no

RA-15 MMTV no no yes

RA-16 MMTV CME yes yes

RA-17 MMTV. . .

DETD . . . The HIV 5'LTR promoter can be deleted and replaced with Moloney virus promoter, MMTV LTR, Actin promoter, myosin promoter and **CMV** promoter.

DETD

Construct	Promoter	poly(A)	Amp <sup>r</sup>
-----------	----------	---------	------------------

LA-1	Moloney	HIV 3'LTR	yes
------	---------	-----------	-----

LA-2	Moloney	SV40	yes
------	---------	------	-----

LA-4	Moloney	SV40	no
LA-5	<b>CMV</b>	HIV 3'LTR	yes
LA-6	<b>CMV</b>	SV40	yes
LA-7	<b>CMV</b>	HIV 3'LTR	no
LA-8	<b>CMV</b>	SV40	no
LA-9	MMTV	HIV 3'LTR	yes
LA-10	MMTV	SV40	yes
LA-11	MMTV	HIV 3'LTR	no
LA-12	MMTV	SV40	no
LA-13	HIV 5' LTR	HIV.	.

DETD . . . the thymidine kinase promoter and polyadenylation site. The HIV env coding region is placed under the regulatory control of the **CMV** promoter and SV40 polyadenylation site. The HIV env coding region was obtained as a 2.3 kb PCR fragment from HIV/3B.

DETD . . . the thymidine kinase promoter and polyadenylation site. The HIV gag/pol coding region is placed under the regulatory control of the **CMV** promoter and SV40 polyadenylation site. The HIV gag/pol coding region was obtained from HIV MN, Genbank sequence MI7449, and includes.

DETD . . . the thymidine kinase promoter and polyadenylation site. The HIV gag/pol coding region is placed under the regulatory control of the **CMV** promoter and SV40 polyadenylation site. The HIV gag/pol coding region was obtained from HIV MN, Genbank sequence MI7449, and includes.

DETD Several safety features are included in pGAGPOL.rev. These include use of the **CMV** promoter and a non-retroviral poly(A) site. Furthermore, deletion of the  $\psi$  sequence limits the ability to package viral RNA. In.

DETD Several safety features are included in pGAGPOL.rev. These include use of the **CMV** promoter and a non-retroviral poly(A) site. Furthermore, tat has been deleted and a 50% deletion of nef yields an "inactive".

DETD Step 4a. Digest with BamHI and ligate with the **CMV** promoter obtained by PCR of pCEP4 (Invitrogen, San Diego Calif.) with primers SEQ ID NO:21 and SEQ ID NO:22.

DETD . . . be any promoter functional in a human cell. It is preferred that the promoter is an SV40 promoter or a **CMV** promoter, preferably a **CMV** immediate early promoter. The polyadenylation signal may be any polyadenylation signal functional in a human cell. It is preferred that. . . separated by non-coding regions. In order to be properly expressed, a coding region must have an initiation codon and a **termination** codon.

DETD . . . be any promoter functional in a human cell. It is preferred that the promoter is an SV40 promoter or a **CMV** promoter, preferably a immediate early **CMV** promoter. The polyadenylation signal may be any polyadenylation signal functional in a human cell. It is preferred that the polyadenylation. . . separated by non-coding regions. In order to be properly expressed, a coding region must have an initiation codon and a **termination** codon.

DETD . . . identical to or substantially similar to epitopes of HIV proteins. The coding sequence is under the regulatory control of the **CMV** immediate early promoter and the SV40 minor polyadenylation signal.

DETD . . . encodes at least one HIV protein or a fragment thereof. The coding sequence is under the regulatory control of the **CMV** immediate early promoter and the SV40 minor polyadenylation signal. The HIV protein is selected from the group consisting of gag.

DETD . . . identical to or substantially similar to epitopes of HIV proteins. The coding sequence is under the regulatory control of the **CMV** immediate early promoter and the SV40 minor polyadenylation signal. The two expression units are encoded in opposite directions of each.

DETD . . . protein or a fragment thereof. Each expression unit comprises a coding sequence that is under the regulatory control of the **CMV** immediate early promoter and the SV40 minor polyadenylation signal. The HIV protein is selected from the group consisting of gag.

DETD Step 9. Cut pCEP4 (Invitrogen) with SalI to release a DNA fragment containing the **CMV** promoter, polylinker, and SV40 poly A site. Purify this fragment and blunt-off with the Klenow fragment of DNA Polymerase I.

DETD . . . fragment obtained in step 9. Isolate plasmid containing the bacterial origin of replication, the Kan<sup>R</sup> gene, the RSV enhancer, the **CMV** promoter, polylinker, and the SV40 poly A site.

DETD . . . coding region of rev containing both the Avail site and the nucleotide encoding amino acid 81. A stop codon causing **termination** of Nef at amino acid position 63 and the 3' coding cloning site, MluI, will be introduced by the 3'.

DETD . . . examples include Senilis viruses, RossRiver virus and Eastern & Western Equine encephalitis. Reovirus: (Medical) Rubeola virus.

Flariviridae Family  
Examples include: (Medical) **dengue**, yellow



encephalitis and tick borne encephalitis  
viruses.

Hepatitis C Virus: (Medical) these viruses are not placed in  
a family yet but are believed to be either a togavirus or a  
**flavivirus**. Most similarity is with togavirus family.

Coronavirus Family:

(Medical and Veterinary)

Infectious bronchitis virus (poultry)

Porcine transmissible gastroenteric virus

(pig)

Porcine. . .

. . . HBV; hepatitis C virus, HCV; human papilloma virus, HPV; Herpes  
simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus,  
**CMV**; Epstein-Barr virus, EBV; rhinovirus; and, coronavirus.

L8 ANSWER 7 OF 11 USPATEFULL on STN

1998:150739 Alphavirus vector constructs.

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**APPLICATION: US 1996-739167 19961030 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. An alphavirus vector construct, comprising a promoter 5' of viral  
cDNA which initiates the synthesis of RNA from the viral cDNA by a  
process of in vitro transcription, followed by a 5' sequence which  
initiates transcription of alphavirus RNA, followed by a nucleotide  
sequence encoding alphavirus non-structural proteins, a viral junction  
region which has been inactivated such that viral transcription of a  
subgenomic fragment is prevented, an internal ribosome entry site or a  
sequence which promotes ribosome readthrough between adjacent reading  
frames, and an alphavirus RNA polymerase recognition sequence.

2. An alphavirus vector construct, comprising a promoter 5' of viral  
cDNA which initiates the synthesis of RNA from the viral cDNA by a  
process of in vitro transcription, followed by a 5' sequence which  
initiates transcription of alphavirus RNA, followed by a nucleotide  
sequence encoding alphavirus non-structural proteins, a viral junction  
region which has been modified such that viral transcription of a  
subgenomic fragment is reduced, an internal ribosome entry site or a  
sequence which promotes ribosome readthrough between adjacent reading  
frames, and an alphavirus RNA polymerase recognition sequence.

3. An alphavirus vector construct, comprising a promoter 5' of viral  
cDNA which initiates the synthesis of RNA from the viral cDNA by a  
process of in vitro transcription, followed by a 5' sequence which  
initiates transcription of alphavirus RNA, followed by a nucleotide  
sequence encoding alphavirus non-structural proteins, a first viral  
junction region which has been inactivated such that viral transcription  
of a subgenomic fragment is prevented, a second viral junction region  
which has been modified such that viral transcription of the subgenomic  
fragment is reduced, an internal ribosome entry site or a sequence which  
promotes ribosome readthrough between adjacent reading frames, and an  
alphavirus RNA polymerase recognition sequence.

4. An alphavirus cDNA vector construct comprising a promoter 5' of viral  
cDNA which initiates the synthesis of RNA from the viral EDNA within a  
cell, followed by a 5' sequence which initiates transcription of  
alphavirus RNA, followed by a nucleotide sequence encoding alphavirus  
non-structural proteins, a viral junction region consisting of (i) all  
active viral junction region, (ii) a viral junction region which has  
been modified such that viral transcription of a subgenomic fragment is  
reduced, and (iii) a viral junction region which has been inactivated  
such that viral transcription of a subgenomic fragment is prevented, and  
an alphavirus RNA polymerase recognition sequence.

5. An alphavirus cDNA vector construct comprising a promoter 5' of viral  
cDNA which initiates the synthesis of RNA from the viral cDNA within a  
cell, followed by a 5' sequence which initiates transcription of  
alphavirus RNA, a nucleotide sequence encoding alphavirus non-structural  
proteins, a first viral junction region which has been inactivated such  
that viral transcription of the subgenomic fragment is prevented,  
followed by a second viral junction region which has been modified such  
that viral transcription of the subgenomic fragment is reduced, and an

6. A vector construct according to any one of claims 1 to 5, further comprising a polyadenylation sequence.
7. A vector construct according to any one of claims 1 to 5 wherein said alphavirus is selected from the group consisting of Venezuelan Equine Encephalitis, Ross River and Semliki Forest viruses.
8. A vector construct according to any one of claims 1 to 5 wherein said alphavirus is Sindbis virus.
9. A vector construct according to any one of claims 1 to 5, further comprising a selected heterologous nucleotide sequence.
10. A vector construct according to claim 9 wherein said vector construct contains a selected heterologous nucleotide sequence ranging in size from about 100 bases to about 8 kb.
11. A vector construct according to claim 9 wherein said selected heterologous nucleotide sequence is a sequence encoding a protein selected from the group consisting of IL-12, IL-15, and GM-CSF.
12. A vector construct according to claim 9 wherein said selected heterologous nucleotide sequence is IL-2.
13. A vector construct according to claim 9 wherein said selected heterologous nucleotide sequence is obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, HBV, HIV, HSV, FeLV, FIV, HTLV-I, HTLV-II, and CMV.
14. A vector construct according to claim 9 wherein said selected heterologous nucleotide sequence is obtained from a hepatitis C virus.
15. A vector construct according to claim 9 wherein said selected heterologous nucleotide sequence is selected from the group consisting of an antisense sequence, a non-coding sense sequence, and a ribozyme sequence.
16. A vector construct according to any one of claims 1 to 5 wherein said vector construct contains no alphavirus structural protein genes.
17. A vector construct according to any one of claims 1 to 5 wherein a selected heterologous nucleotide sequence is located downstream from said viral junction region.
18. A vector construct according to claim 2 or 5 wherein a selected heterologous nucleotide sequence is located downstream from said second viral junction region.
19. A vector construct according to claim 9 wherein said selected heterologous nucleotide sequence is located within a nucleotide sequence encoding alphavirus non-structural proteins.
20. A vector construct according to claim 1, 3, 4, or 5 wherein said inactivated viral junction region consists of the nucleotide sequence as shown in Sequence ID: No. 1, from nucleotide number 7579, to nucleotide 7597.
21. A vector construct according to claim 3 or 5 further comprising an adenovirus L3 gene or CMV H301 gene.
22. A vector construct according to claim 3 or 5 further comprising a non-alphavirus packaging sequence.
23. The vector construct according to claim 3 or 5 further comprising a 3' transcription **termination** site.
24. The vector construct according to claim 23 wherein said transcription **termination** site is a **termination**/polyadenylation sequence.
25. A recombinant alphavirus particle which, upon introduction into a BHK cell, produces an infected cell which is viable at least 72 hours after infection.
26. A recombinant alphavirus particle which, upon introduction into a BHK cell, produces an infected cell which is viable at least 72 hours after infection, said particle also carrying a vector construct which directs the expression of at least one antigen or modified form thereof in target cells infected with the alphavirus particle, wherein said antigen or modified form thereof stimulates an immune response within an animal.

27. A recombinant alphavirus particle according to claim 26 wherein the expressed antigen elicits an immune response selected from the group consisting of a cell-mediated immune response, a HLA Class I- restricted immune response, and a HLA Class II-restricted immune response.
28. A cell infected with a recombinant alphavirus particle according to any one of claims 25 to 27.
29. A cell according to claim 27 wherein said cell is a mammalian cell.
30. A packaging cell line which inducibly expresses alphavirus structural proteins, and which, upon introduction of an alphavirus vector construct, produces recombinant alphavirus particles.
31. A packaging cell line according to claim 30 derived from mammalian cells.
32. A packaging cell line according to claim 30 derived from mammalian cells.
33. A packaging cell line according to claim 32 derived from insect cells.
34. A packaging cell line according to claim 32 wherein said insect cells are mosquito cells.
35. A packaging cell line according to claim 30 wherein the packaging cell line, upon introduction of a vector construct, produces alphavirus particles which infect human cells.
36. A packaging cell line according to claim 30 wherein an alphavirus inhibitory protein is not produced.
37. A packaging cell line suitable for packaging and production of an alphavirus vector, wherein the packaging cell line comprises an expression cassette which directs the expression of VSV-G.
38. A packaging cell line according to claim 37, further comprising an expression cassette which directs the expression of one or more alphavirus structural proteins.
39. A packaging cell line according to claims 30 or 37 wherein said cell line expresses a gene product which suppresses apoptosis.
40. The packaging cell according to claim 39 wherein said gene product is encoded by a gene selected from the group consisting of bcl-2 oncogene, adenovirus E1B gene encoding a 19-kD protein, herpes simplex virus type 1  $\gamma$ 34.5 gene, and AcMNPV baculovirus p35 gene.
41. An alphavirus producer cell line, comprising a packaging cell line according to claim 30, and an alphavirus vector construct or alphavirus cDNA vector construct, wherein said producer cell line produces recombinant alphavirus particles.
42. An alphavirus producer cell line according to claim 41 wherein said recombinant alphavirus particles infect human cells.
43. An alphavirus producer cell line according to claim 41 wherein said producer cell line inducibly produces recombinant alphavirus particles.
44. An alphavirus producer cell line according to claim 41 wherein said producer cell line produces recombinant alphavirus particles in response to a differentiation state of said producer cell line.
45. A producer cell line suitable for packaging and production of a recombinant alphavirus particle, wherein the producer cell line comprises an expression cassette which directs the expression of gag/pol, an expression cassette which directs the expression of env, and an alphavirus vector construct containing a retroviral packaging sequence.
46. A producer cell line suitable for packaging and production of a recombinant alphavirus particle, wherein the producer cell line comprises one or more expression cassettes which direct the expression of non-alphaviral structural proteins, and an alphavirus vector construct comprising a packaging sequence corresponding to a virus from which the non-alphaviral structural proteins are derived.
47. A method for producing recombinant alphavirus particles from a packaging cell line, the method comprising introducing an alphavirus vector construct into a packaging cell line according to any one of claims 30 to 41 by a process selected from the group consisting of (i)

initiation system, (ii) transfection of the packaging cell line with RNA transcribed in vitro from an alphavirus vector construct, and (iii) infection of the packaging cell line with recombinant alphavirus particles.

**AI US 1996-739167 19961030 (8)**

SUMM . . . transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**.

SUMM . . . transcription of the subgenomic fragment is reduced, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**.

SUMM . . . transcription of the subgenomic fragment is reduced, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**.

SUMM . . . cell, the vector construct being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**.

SUMM . . . the vector construct being capable of expressing a heterologous ribonucleic acid sequence, and a 3' DNA sequence which controls transcription **termination**.

SUMM . . . from cDNA is selected from the group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40 promoter, and the **CMV** promoter. Within further embodiments, the eukaryotic layered vector initiation systems further comprise a polyadenylation sequence.

SUMM . . . group consisting of influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and **CMV**. Within one preferred embodiment, the heterologous sequence obtained from HPV encodes a protein selected from the group consisting of E5. . .

SUMM . . . are complementary to influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and **CMV** sequences.

SUMM . . . 5C distal, SV40, heat shock protein 65, heat shock protein 70, Py, RSV, BK, JC, MuLV, MMTV, alphavirus junction region, **CMV** and VA1RNA.

DETD . . . reduce viral transcription of the subgenomic fragment, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**. The vector may also include nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus. . .

DETD . . . sequence encoding one or more proteins, said proteins preferably comprising alphavirus structural protein(s). Optionally, the expression cassette may include transcription **termination**, splice recognition, and polyadenylation addition sites. Preferred promoters include the **CMV**, MMTV, MoMLV, and adenovirus VA1RNA promoters. In addition, the expression cassette may contain selectable markers such as Neo, SV2 Neo. . .

DETD . . . promoters, such as, for example, the  $\beta$ -galactosidase promoter, trpE promoter, lacZ promoter, T7 promoter, T3 promoter, SP6 promoter, SV40 promoter, **CMV** promoter, and MoMLV LTR.

DETD 4. THE **CMV** H301 GENE

DETD . . . the human immunoglobulin heavy-chain binding protein, and a synthetic sequence of at least 15 bps corresponding in part to the **Kozak** consensus sequence for efficient translational initiation. Although not described in detail here, these signals which affect translation initiation can also. . .

DETD As noted above, the alphavirus cDNA vector construct also includes a 3' sequence which controls transcription **termination**. A representative example of such a sequence is set forth in more detail in Examples 2 and 3.

DETD . . . vector may be selected to hybridize to any pathogen-specific RNA, for instance target cells infected by pathogens such as HIV, **CMV**, HBV, HPV and HSV.

DETD . . . that any truncation leave intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational **termination** codons may be introduced downstream of the immunogenic region. Insertion of **termination** codons will prematurely terminate protein expression, thus preventing expression of the transforming portion of the protein.

DETD . . . "HCV", respectively), Human Papilloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, HTLV I, HTLV II and Cytomegalovirus ("**CMV**"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which. . .

DETD . . . cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**. Briefly, such eukaryotic layered vector initiation systems provide a two-stage or "layered" mechanism which controls expression of heterologous nucleotide sequences. . . promoter which is capable of initiating the 5' synthesis of RNA from cDNA (e.g., a 5' promoter), a 3' transcription **termination** site, as well as one or more splice sites and/or a polyadenylation site, if desired. Representative promoters suitable for use. . . Mouse polyoma

promoters, MMTV promoter, alphavirus junction region, **CMV** promoter, Adenovirus VA1RNA, rRNA promoter, tRNA methionine promoter, CaMV 35S promoter, nopaline synthetase promoter, and the lac promoter. The second. . .

DETD . . . example, vector systems derived from viruses of the following families: Picornaviridae (e.g., poliovirus, rhinovirus, coxsackieviruses), Caliciviridae, Togaviridae (e.g. alphavirus, rubella), **Flaviviridae** (e.g., yellow fever), Coronaviridae (e.g., HCV, TGEV, IBV, MHV, BCoV), Bunyaviridae, Arenaviridae, Retroviridae (e.g., RSV, MoMLV, HIV, HTLV), hepatitis delta. . .

DETD . . . transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**. Within various embodiments, the viral junction region may be modified, such that viral transcription of the subgenomic fragment is merely. . .

DETD . . . auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV HTLV I, HTLV II, **CMV**, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease.

DETD . . . cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**. Within one embodiment, such constructs may be constructed of the following ordered elements: a 5' eukaryotic promoter capable of initiating. . . alphavirus non-structural proteins, a viral junction region, a heterologous sequence, an alphavirus RNA polymerase recognition sequence, and a 3' transcription **termination**/polyadenylation signal sequence. Such alphavirus cDNA expression vectors may also include intervening sequences (introns), which are spliced from the pre-RNA in. . .

DETD . . . occurs from the pKSSINBVd1JR construct. Junction region changes at Sindbis nts 7600 and 7602 are downstream of the NSP 4 **termination** codon and upstream of the structural proteins initiation codon.

DETD . . . are observed among the eight alphaviruses sequenced (Semliki Forest virus, Middleburg virus, Ross River virus, O'Nyong Nyong virus, Eastern Equine **Encephalitis virus**, Western Equine **Encephalitis virus**, and Venezuelan Equine **Encephalitis virus**) are given below:

DETD In order to insert the luciferase gene into the pVGELVIS-SINBV vector, the SV40 intron and transcription **termination** sequences at the 3'-end of luciferase must be removed so that when the pre-RNA, transcribed from the plasmid DNA luciferase. . .

DETD . . . expression, is enhanced by several modifications to the pVGELVIS-SINBV-luc vector. These modifications include alternate RNA polymerase II promoters and transcription **termination** signals, the addition of intron sequences and ribozyme processing signals in the vector construct, and substitution with a smaller plasmid. . .

DETD The transcription **termination** signals from the SV40 early region or Bovine growth hormone are inserted between the Sac I and Eco RI sites of pBGS131. The SV40 nts between viral nts 2643 to 2563 containing the early region transcription **termination** sequences are isolated by PCR amplification using the primer pair shown below and the pBR322/SV40 plasmid (ATCC #45019) as template.

DETD The Bovine growth hormone transcription **termination** sequences are isolated by PCR amplification using the primer pair shown below and the pCDNA3 plasmid (Invitrogen) as template.

DETD In additional modifications to the ELVIS vector, the transcription **termination** sequences are fused directly to the 3'-end Sindbis sequences, resulting in deletion of the polyadenylate tract; or alternatively the antigenomic. . . ribozyme sequence of hepatitis delta virus (HDV) is inserted between the 3'-polyadenylate tract of the ELVIS vector and the transcription **termination** signals.

DETD In the second vector 3'-end configuration, the SV40 or BGH transcription **termination** signals are fused directly to the 3'-end of the ELVIS vector corresponding to Sindbis nt 11,700 and the polyadenylate tract. . .

DETD . . . a polyadenylate tract are combined with the plasmid constructions containing the HDV ribozyme sequence and the SV40 or BGH transcription **termination** signals. This construction corresponds to the insertion of pKSSINBV and pKSSINBV-luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids. Alternatively,. . . terminating precisely at the viral 3'end corresponding to viral nt 11,700 are linked directly to the SV40 or BGH transcription **termination** signals. This construction corresponds to the insertion of pKSSINBVd1A and pKSSINBVd1A-luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids.

DETD Using the same overlapping PCR approach, the **CMV** promoter is positioned at the 5' viral end such that transcription initiation results in the addition of a single non-viral nucleotide at the Sindbis 5' end. Amplification of the **CMV** promoter in the first primary PCR reaction is accomplished in a reaction containing the pCDNA3 plasmid and the following primer. . .

DETD Forward primer: pCBgl233F (buffer sequence/Bgl II recognition sequence/**CMV** promoter nts 1-22):

DETD Forward primer: CMVSIN1F (**CMV** pro nts 1124-1142/SIN nts 1-20):

DETD Forward primer: pCBgl233F (buffer sequence/Bgl II recognition sequence/**CMV** promoter nts 1-22):

DETD . . . plasmid DNA (FIG. 22A). The data also demonstrate that direct linkage between the Sindbis virus 3'-end and two different transcription **termination**/polyadenylation signals, resulting in deletion of the synthetic A<sub>25</sub> tract, decreased the activity of the DNA vector by more than three. . .

DETD . . . of in vitro synthesized transcripts, the cDNA is exchanged into a vector containing an RNA polymerase II promoter and transcription **termination**/polyadenylation sequence, as described in Example 2. For plant applications, such promoter and **termination** sequences are chosen from the appropriate plant systems (e.g., CaMV 35S promoter (Guilley et al., Cell 30:763-773, 1982), and nopaline synthase promoter and transcription **termination** sequence (Sanders et al., Nucleic Acids Res. 15:1543-1558). Vector constructs derived from these infectious genomic cDNA clones is subsequently accomplished. . .

DETD . . . downstream of the disabled junction region in the pKSSINBVdlJR vector, which allows ribosomal scanning in genomic mRNA from non-structural gene **termination** to the heterologous genes. The heterologous proteins are expressed from genomic length mRNA by ribosomal scanning. This extends the life. . .

DETD . . . et al., Gene 108:167-174, 1991). The sequences surrounding the ATG start codon from bases -9 to +1 conform to the **Kozak** consensus sequence for efficient translational initiation (**Kozak**, Cell 44:283-292, 1986). Where possible, the 3' terminal nucleotide corresponding to the carboxy terminal amino acid is changed to T. . .

DETD . . . gpt, for the current neomycin resistance, or other promoter elements, for example Drosophila metallothionein or hsp 70, for the current **CMV**, MuLV, and SV40 promoters, may be readily accomplished given the disclosure provided herein.

DETD . . . map, respectively, to the authentic AUG translational start and UGA translational stop sites, including the surrounding nucleotides corresponding to the **Kozak** consensus sequence for efficient translational initiation at Sindbis nt 7638. The forward primer is complementary to Sindbis nts 7638-7661, and. . .

DETD . . . structural protein gene mRNA, the alphavirus structural protein gene sequences, the 3'-end alphavirus sequences required for replication, and a transcription **termination**/polyadenylation sequence. Because of an upstream open-reading frame which ends in translation **termination** codons prior to the AUG start site of the structural protein genes, expression of the alphavirus structural proteins can occur. . .

DETD In addition to their respective complementarities, primer SIN276-SPE contains a flanking UAA translation **termination** codon and SpeI recognition sequence at its 5' end. PCR amplification of the fragment containing Sindbis 5'-end sequences fused to. . .

DETD Modifications of the **CMV** promoter/antisense-Sindbis structural protein vector also can be constructed using other viral, cellular, or insect-based promoters. Using common molecular biology techniques known in the art, the **CMV** promoter can be switched out of the Invitrogen pCDNA3 vector and replaced by promoters such as those listed previously. Other. . . use of longer or shorter HDV or other catalytic ribozyme sequences for transcript processing, the substitution of a precise transcription **termination** signal for the catalytic ribozyme sequence, or the antisense expression of structural protein gene cassettes using any downstream sequence recognized. . .

DETD . . . vector and the Astrovirus expression cassette, between the Astrovirus junction region and structural gene ATG, and between the structural gene **termination** codon and the Astrovirus 3' end. In order to generate wild type virus, this dual recombination event would have to. . .

DETD . . . glycoproteins E1 and/or E2 from other alphaviruses or their variants is performed to alter tissue tropism. For example, Venezuelan equine **encephalitis virus** (VEE) is an alphavirus which exhibits tropism for cells of lymphoid origin, unlike its Sindbis virus counterpart. Therefore, Sindbis-derived vector. . .

DETD . . . is amplified with a primer pair whose 5'-ends map, respectively, to the authentic AUG translational start site, including the surrounding **Kozak** consensus sequence, and UGA translational stop site. The forward primer is complementary to VEE nucleotides 7553-7579, and the reverse primer. . .

DETD . . . Madison, Wis.) by digestion of pCDNA3 with Apa I and EcoRV followed by GENECLEAN.TM. purification. The resulting construct, containing a **CMV** promoter which transcribes an antisense reporter cassette RNA of the configuration Sindbis 3'-end sequence/LacZ gene/junction region/Sindbis 5'-end sequence/HDV ribozyme, is. . .

DETD . . . vector. The re-ligated construct contains an antisense LacZ gene and a 3' Sindbis replicase protein recognition sequence downstream from a **CMV** promoter. The resulting construct is known as pCDNAaLacZ-3'Sin. The construct is transfected into BHK cells and utilized as described previously.

measure CTL responses to other non-HIV antigens such as mouse **CMV**(MCMV).  
 DETD Subgenomic mRNA is synthesized in these vectors, which serves as a translational template for the Ad E3 and **CMV** H301 genes. Thus, in these constructions, functional HPV 16 antisense and hairpin ribozyme palliatives will be present on the levels. . . .  
 DETD . . . the E6/E7 or L1 proteins. In these vectors, it would not be desired to include the immunoregulatory Ad E3 or **CMV** H301 genes.  
 DETD . . . among the group described in Example 5, and the therapeutic palliative. Ordered insertion of the hairpin ribozyme, Ad E3 or **CMV** H301, IRES, and heterologous gene of interest components along the multiple cloning sequence located in the vector between the vector. . .  
 DETD The coding region and transcriptional **termination** signals of HSV-1 thymidine kinase gene (HSV-TK) are isolated as a 1.8 kb Bgl II/Pvu II fragment from plasmid 322TK. . . .  
 . . . selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, HBV, HIV, HSV, FeLV, FIV, HTLV-I, HTLV-II, and **CMV**.

21. A vector construct according to claim 3 or 5 further comprising an adenovirus L3 gene or **CMV** H301 gene.

23. The vector construct according to claim 3 or 5 further comprising a 3' transcription **termination** site.

24. The vector construct according to claim 23 wherein said transcription **termination** site is a **termination**/polyadenylation sequence.

L8 ANSWER 8 OF 11 USPATFULL on STN

1998:122388 Genetic immunization.

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US 5817637 19981006

**APPLICATION: US 1997-783818 19970113 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. An pharmaceutical immunizing kit comprising: a) a first inoculant comprising: i) a pharmaceutically acceptable carrier or diluent; and, ii) a first nucleic acid molecule comprising a nucleotide sequence that encodes at least one HIV protein operatively linked to regulatory sequences; wherein said nucleotide sequence is capable of being expressed in human cells; b) a second inoculant comprising: i) a pharmaceutically acceptable carrier or diluent; and, ii) a second nucleic acid molecule comprising a nucleotide sequence that encodes at least one HIV protein operatively linked to regulatory sequences; wherein said nucleotide sequence is capable of being expressed in human cells; wherein said first nucleic acid molecule is not identical to said second nucleic acid molecule and, taken together, said first nucleic acid molecule and said second nucleic acid molecule encode HIV proteins gag, pol and env; and c) a third inoculant comprising bupivacaine.

2. A pharmaceutical composition comprising: a) a compound selected from the group consisting of: bupivacaine, mepivacaine, lidocaine, procaine, carbocaine and methyl bupivacaine; and b) a DNA molecule that comprises a DNA sequence that encodes an antigen; wherein said DNA sequence operatively linked to regulatory sequences which control the expression of said DNA sequence.

3. The pharmaceutical composition of claim 2 wherein said composition comprises bupivacaine.

4. The pharmaceutical composition of claim 2 wherein said DNA molecule is a plasmid.

5. The pharmaceutical composition of claim 2 wherein said DNA sequence encodes a variable region of a T cell receptor.

6. The pharmaceutical composition of claim 2 wherein said DNA sequence encodes a pathogen antigen.

7. The pharmaceutical composition of claim 6 wherein said DNA sequence encodes an antigen from an intracellular pathogen.

8. The pharmaceutical composition of claim 7 wherein said antigen is a viral antigen.

9. The pharmaceutical composition of claim 8 wherein said pathogen is a virus selected from the group consisting of: human immunodeficiency virus, HIV; human T cell leukemia virus, HTLV; influenza virus; hepatitis A virus; hepatitis B virus; hepatitis C virus; human papilloma virus, HPV; Herpes simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus, **CMV**; Epstein-Barr virus, EBR; rhinovirus; and, coronavirus.
10. The pharmaceutical composition of claim 9 wherein said pathogen is Herpes simplex 2 virus, HSV2.
11. The pharmaceutical composition of claim 9 wherein said pathogen is Hepatitis B virus, HBV.
12. The pharmaceutical composition of claim 9 wherein said pathogen is human T cell leukemia virus, HTLV.
13. The pharmaceutical composition of claim 9 wherein said pathogen is a human immunodeficiency virus.
14. The pharmaceutical composition of claim 2 wherein said DNA sequence encodes a hyperproliferative disease associated protein.
15. The pharmaceutical composition of claim 13 wherein said hyperproliferative disease is cancer.
16. The pharmaceutical composition of claim 14 wherein said hyperproliferative disease is a lymphoma.
17. The pharmaceutical composition of claim 14 wherein said hyperproliferative disease is a melanoma.
18. a method of immunizing an individual against an antigen comprising administering to tissue of said individual's body, a) a compound selected from the group consisting of bupivacaine, mepivacaine, lidocaine, procaine, carbocaine and methyl bupivacaine, and b) a DNA molecule that comprises a DNA sequence that encodes said antigen, said DNA sequence operatively linked to regulatory sequences which control the expression of said DNA sequence; wherein said DNA molecule is taken up by cells, said DNA sequence is expressed in said cells and an immune response is generated against said antigen.
19. The method of claim 18 wherein said compound is bupivacaine.
20. The method of claim 18 wherein said DNA molecule is a plasmid.
21. The method of claim 18 wherein said immune response generated against said antigen is an immune response against a pathogen antigen.
22. The method of claim 21 wherein said pathogen is an intracellular pathogen.
23. The method of claim 22 wherein said pathogen is a virus selected from the group consisting of: human immunodeficiency virus, HIV; human T cell leukemia virus, HTLV; influenza virus; hepatitis a virus; hepatitis B virus; hepatitis C virus; human papilloma virus, HPV; Herpes simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus, **CMV**; Epstein-Barr virus, EBR; rhinovirus; and, coronavirus.
24. The method of claim 23 wherein said pathogen is HIV and said DNA molecule comprises a DNA sequence that encodes an HIV antigen.
25. The method of claim 18 wherein said immune response generated against said antigen is an immune response against a hyperproliferative disease-associated protein.
26. The method of claim 25 wherein said hyperproliferative disease-associated protein is selected from the group consisting of: protein products of oncogenes myb, myc, fyn, ras, src, neu and trk; protein products of translocation gene bcr/abl; P53; variable regions of antibodies made by B cell lymphomas; and variable regions of T cell receptors of T cell lymphomas.
27. The method of claim 18 wherein said immune response generated against said antigen is an immune response against an autoimmune disease-associated protein.
28. The method of claim 27 wherein said autoimmune disease-associated protein is selected from the group consisting of: variable regions of antibodies involved in B cell mediated autoimmune disease; and variable regions of T cell receptors involved in T cell mediated autoimmune disease.



29. The method of claim 18 wherein said bupivacaine and a DNA molecule are administered subcutaneously.

30. The method of claim 18 wherein said bupivacaine and a DNA molecule are administered intramuscularly, intraperitoneally, intravenously, intraarterially, intraocularly, orally transdermally and/or by inhalation.

31. The method of claim 18 wherein said bupivacaine and a DNA molecule are administered intradermally.

32. The method of claim 31 wherein said immune response generated against said antigen is an immune response against a pathogen antigen.

33. The method of claim 31 wherein said immune response generated against said antigen is an immune response against a hyperproliferative disease-associated protein.

34. The method of claim 31 wherein said immune response generated against said antigen is an immune response against an autoimmune disease-associated protein.

**AI US 1997-783818 19970113 (8)**

DETD . . . the individual. Regulatory elements for DNA include a promoter and a polyadenylation signal. In addition, other elements, such as a **Kozak** region, may also be included in the genetic construct.

DETD . . . the DNA or RNA molecule that comprises a nucleotide sequence which encodes the target protein and which includes. initiation and **termination** signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of. . .

DETD . . . Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (**CMV**) such as the **CMV** immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as. . .

DETD . . . but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from **CMV**, RSV and EBV.

DETD . . . be operably linked to the nucleotide sequence that encodes the target protein. Accordingly, it is necessary for the initiation and **termination** codons to be in frame with the coding sequence.

DETD . . . and a polyadenylation signal. The promoter may be selected from the group consisting of: HIV LTR, human Actin, human Myosin, **CMV**, RSV, Moloney, MMTV, human Hemoglobin, human muscle creatine and EBV. The enhancer may be selected from the group consisting of: human Actin, human Myosin, **CMV**, RSV, human Hemoglobin, human muscle creatine and EBV. The polyadenylation signal may be selected from the group consisting of: LTR. . .

DETD A region from just upstream of the unique PflMI site to just after the vif **termination** codon was amplified via PCR using primers that introduced a non-conservative amino acid change (glu→val) at amino acid 22 of. . .

DETD . . . HXB2 was amplified via PCR and cloned into the expression vector pCND4/neo (Invitrogen). This plasmid drives envelope production through the **CMV** promoter.

DETD . . . expression is MMTV LTR. The promoter may be deleted and replaced with Actin promoter, myosin promoter, HIV LTR promoter and **CMV** promoter.

DETD . . . Actin no yes no

RA-3 Actin no no yes

RA-4 Actin CME yes yes

RA-5 Actin CME yes no

RA-6 Actin CME no yes

RA-7 **CMV** no yes yes

RA-8 **CMV** no yes no

RA-9 **CMV** no no yes

RA-10 **CMV** CME yes yes

RA-11 **CMV** CME yes no

RA-12 **CMV** CME no yes

RA-13 MMTV no yes yes

RA-14 MMTV no yes no

RA-15 MMTV no no yes

RA-16 MMTV CME yes yes

RA-17 MMTV. . .

DETD . . . to be introduced by vaccination. A region from just upstream of the unique PflMI site to just after the vif **termination** codon was amplified via PCR using primers that introduced a non-conservative amino acid change (glu→val) at amino acid 22 of. . .

DETD . . . HIV 5' LTR promoter can be deleted and replaced with Moloney virus promoter, MMTV LTR, Actin promoter, myosin promoter and **CMV** promoter.

Construct	Promoter	poly(A)	Amp <sup>r</sup>
LA-1	Moloney	HIV 3'LTR	yes
LA-2	Moloney	SV40	yes
LA-3	Moloney	HIV 3'LTR	no
LA-4	Moloney	SV40	no
LA-5	<b>CMV</b>	HIV 3'LTR	yes
LA-6	<b>CMV</b>	SV40	yes
LA-7	<b>CMV</b>	HIV 3'LTR	no
LA-8	<b>CMV</b>	SV40	no
LA-9	MTTV	HIV 3'LTR	yes
LA-10	MTTV	SV40	yes
LA-11	MTTV	HIV 3'LTR	no
LA-12	MTTV	SV40	no
LA-13	HIV 5' LTR	HIV	
DETD	. . . the thymidine kinase promoter and polyadenylation site. The HIV env coding region is placed under the regulatory control of the <b>CMV</b> promoter and SV40 polyadenylation site. The HIV env coding region was obtained as a 2.3 kb PCR fragment from HIV/3B, . . .		
DETD	. . . the thymidine kinase promoter and polyadenylation site. The HIV gag/pol coding region is placed under the regulatory control of the <b>CMV</b> promoter and SV40 polyadenylation site. The HIV gag/pol coding region was obtained from HIV MN, Genebank sequence MI7449, and includes. . .		
DETD	. . . the thymidine kinase promoter and polyadenylation site. The HIV gag/pol coding region is placed under the regulatory control of the <b>CMV</b> promoter and SV40 polyadenylation site. The HIV gag/pol coding region was obtained from HIV MN, Genebank sequence MI7449, and includes. . .		
DETD	Several safety features are included in pGAGPOL.rev. These include use of the <b>CMV</b> promoter and a non-retroviral poly(A) site. Furthermore, deletion of the $\psi$ sequence limits the ability to package viral RNA. In. . .		
DETD	Several safety features are included in PGAGPOL.rev. These include use of the <b>CMV</b> promoter and a non-retroviral poly(A) site. Furthermore, tat has been deleted and a 50% deletion of nef yields an "inactive". . .		
DETD	Step 4a. Digest with BamHI and ligate with the <b>CMV</b> promoter obtained by PCR of pCEP4 (Invitrogen, San Diego, Calif.) with primers SEQ ID NO.:27 and SEQ ID NO.:28.		
DETD	. . . be any promoter functional in a human cell. It is preferred that the promoter is an SV40 promoter or a <b>CMV</b> promoter, preferably a <b>CMV</b> immediate early promoter. The polyadenylation signal may be any polyadenylation signal functional in a human cell. It is preferred that. . . separated by non-coding regions. In order to be properly expressed, a coding region must have an initiation codon and a <b>termination</b> codon.		
DETD	. . . be any promoter functional in a human cell. It is preferred that the promoter is an SV40 promoter or a <b>CMV</b> promoter, preferably a immediate early <b>CMV</b> promoter. The polyadenylation signal may be any polyadenylation signal functional in a human cell. It is preferred that the polyadenylation. . . separated by non-coding regions. In order to be properly expressed, a coding region must have an initiation codon and a <b>termination</b> codon.		
DETD	. . . identical to or substantially similar to epitopes of HIV proteins. The coding sequence is under the regulatory control of the <b>CMV</b> immediate early promoter and the SV40 minor polyadenylation signal.		
DETD	. . . encodes at least one HIV protein or a fragment thereof. The coding sequence is under the regulatory control of the <b>CMV</b> immediate early promoter and the SV40 minor polyadenylation signal. The HIV protein is selected from the group consisting of gag, . . .		
DETD	. . . identical to or substantially similar to epitopes of HIV proteins. The coding sequence is under the regulatory control of the <b>CMV</b> immediate early promoter and the SV40 minor polyadenylation signal. The two expression units are encoded in opposite directions of each. . .		
DETD	. . . protein or a fragment thereof. Each expression unit comprises a coding sequence that is under the regulatory control of the <b>CMV</b> immediate early promoter and the SV40 minor polyadenylation signal. The HIV protein is selected from the group consisting of gag, . . .		
DETD	. . . examples include Senilis viruses, RossRiver virus and Eastern & Western Equine*** encephalitis. Reovirus: (Medical) Rubella virus.		
Flariviridae	Family Examples include: (Medical) <b>dengue</b> , yellow fever, Japanese encephalitis, St. Louis encephalitis and tick borne encephalitis viruses.		
Hepatitis C Virus:	(Medical) these viruses are not placed in a family yet but are believed to be either a togavirus or a <b>flavivirus</b> . Most similarity is with togavirus family.		

(Medical and Veterinary)  
Infectious bronchitis virus (poultry)  
Porcine transmissible gastroenteric virus  
(pig)  
Porcine. . .

. . . B virus; hepatitis C virus; human papilloma virus, HPV; Herpes  
simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus,  
**CMV**; Epstein-Barr virus, EBR; rhinovirus; and, coronavirus.

. . . B virus; hepatitis C virus; human papilloma virus, HPV; Herpes  
simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus,  
**CMV**; Epstein-Barr virus, EBR; rhinovirus; and, coronavirus.

L8 ANSWER 9 OF 11 USPTAFULL on STN

1998:119004 Eukaryotic layered vector initiation systems.

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US 5814482 19980929

**APPLICATION: US 1996-739158 19961030 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A method of stimulating in an animal an immune response to an antigen  
comprising introducing into susceptible target cells a eukaryotic  
layered vector initiation system comprising a eukaryotic promoter 5' of  
viral cDNA which initiates within said cell the 5' to 3' synthesis of  
RNA from said cDNA, wherein said RNA comprises a vector construct which  
autonomously amplifies in said cell and expresses a heterologous nucleic  
acid sequence, wherein said heterologous nucleic acid sequence encodes  
an antigen or modified form thereof which stimulates an immune response  
within an animal.

2. A method according to claim 1 wherein the target cells are infected  
in vivo.

3. A method according to claim 1 wherein the expressed antigen elicits  
an immune response selected from the group consisting of cell-mediated  
immune response, an HLA class I-restricted immune responses, and an HLA  
class II-restricted immune response.

4. The method according to claim 1, wherein said promoter is a DNA  
promoter of RNA synthesis.

5. The method according to claim 1, wherein said vector construct which  
autonomously amplifies in a cell comprises a sequence which initiates  
transcription of alphavirus RNA following the 5' promoter, a nucleic  
acid sequence which encodes alphavirus nonstructural proteins, an  
alphavirus RNA polymerase recognition sequence, and a 3' polyadenylate  
tract.

6. The method according to claim 1, further comprising a transcription  
**termination** sequence.

7. The method according to claim 1, wherein said vector construct which  
autonomously amplifies in a cell is derived from a virus selected from  
the group consisting of poliovirus, rhinovirus, coxsackievirus, rubella,  
yellow fever, RSV, MoMI.V, and Astrovirus.

8. The method according to claim 1, wherein said vector construct which  
autonomously amplifies in a cell is derived from a virus selected from  
the group consisting of tobamoviruses, potyviruses, and bromoviruses.

9. The method according to claim 1, wherein said promoter is selected  
from the group consisting of the MoMLV promoter, metallothionein  
promoter, glucocorticoid promoter, SV40 promoter, CaMV 35S promoter,  
nopaline synthetase promoter, and the **CMV** promoter.

10. The method according to claim 1, wherein said heterologous nucleic  
acid sequence is obtained from a virus selected from the group  
consisting of influenza virus, respiratory syncytial virus, IIPV, EBV,  
HIV, FeLV, FIV, Hantavirus, HTLV, HTLV, II, and **CMV**.

11. The method according to claim 1, wherein said heterologous nucleic  
acid sequence is obtained from HBV.

12. The method according to claim 1, wherein said heterologous nucleic  
acid sequence is obtained from HCV.

13. The method according to claim 1, wherein said heterologous nucleic acid sequence is obtained from HSV.

14. A eukaryotic layered vector initiation system, comprising a eukaryotic promoter 5' of viral cDNA which initiates within a susceptible target cell the 5' to 3' synthesis of RNA from said cDNA, wherein said RNA comprises a vector construct which autonomously amplifies in a cell and expresses a heterologous nucleic acid sequence which encodes an antigen or modified form thereof that stimulates an immune response within an animal.

15. A eukaryotic layered vector initiation system according to claim 14 wherein the expressed antigen elicits an immune response selected from the group consisting of a cell-mediated immune response, an HLA class I-restricted immune response, and an HLA class II-restricted immune response.

16. The eukaryotic layered vector initiation system according to claim 14 wherein said promoter is a DNA promoter of RNA synthesis.

17. The eukaryotic layered vector initiation system according to claim 14 wherein said vector construct which autonomously amplifies in a cell comprises a sequence which initiates transcription of alphavirus RNA following the 5' promoter, a nucleic acid sequence which encodes alphavirus nonstructural proteins, an alphavirus RNA polymerase recognition sequence, and a 3' polyadenylate tract.

18. The eukaryotic layered vector initiation system according to claim 14 further comprising a transcription **termination** sequence.

19. The eukaryotic layered vector initiation system according to claim 14 wherein said vector construct which autonomously amplifies in a cell is derived from a virus selected from the group consisting of poliovirus, rhinovirus, coxsackievirus, rubella, yellow fever, RSV, MoMLV, and Astrovirus.

20. The eukaryotic layered vector initiation system according to claim 14 wherein said vector construct which autonomously amplifies in a cell is derived from a virus selected from the group consisting of tobamoviruses, potyviruses, and bromoviruses.

21. The eukaryotic layered vector initiation system according to claim 14 wherein said promoter is selected from the group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40 promoter, CaMV 35S promoter, nopaline synthetase promoter, and the **CMV** promoter.

22. The eukaryotic layered vector initiation system according to claim 14 wherein said heterologous nucleic acid sequence is obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, EBV, HIV, FeLV, FIV, Hantavirus, HTLV I, HTLV II, and **CMV**.

23. The eukaryotic layered vector initiation system according to claim 14 wherein said heterologous nucleic acid sequence is obtained from HBV.

24. The eukaryotic layered vector initiation system according to claim 14 wherein said heterologous nucleic acid sequence is obtained from HCV.

25. The eukaryotic layered vector initiation system according to claim 14 wherein said heterologous nucleic acid sequence is obtained from HSV.

**AI US 1996-739158 19961030 (8)**

SUMM . . . transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**.

SUMM . . . transcription of the subgenomic fragment is reduced, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**.

SUMM . . . transcription of the subgenomic fragment is reduced, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**.

SUMM . . . cell, the vector construct being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**.

SUMM . . . the vector construct being capable of expressing a heterologous ribonucleic acid sequence, and a 3' DNA sequence which controls transcription **termination**.

SUMM . . . from cDNA is selected from the group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40 promoter, and the **CMV** promoter. Within further embodiments, the eukaryotic layered vector initiation systems further comprise a

SUMM . . . group consisting of influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and **CMV**. Within one preferred embodiment, the heterologous sequence obtained from HPV encodes a protein selected from the group consisting of E5, . . .

SUMM . . . are complementary to influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and **CMV** sequences.

SUMM . . . 5C distal, SV40, heat shock protein 65, heat shock protein 70, Py, RSV, BK, JC, MuLV, MMTV, alphavirus junction region, **CMV** and VA1RNA.

DETD . . . reduce viral transcription of the subgenomic fragment, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**. The vector may also include nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, . . .

DETD . . . sequence encoding one or more proteins, said proteins preferably comprising alphavirus structural protein(s). Optionally, the expression cassette may include transcription **termination**, splice recognition, and polyadenylation addition sites. Preferred promoters include the **CMV**, MMTV, MoMLV, and adenovirus VA1RNA promoters. In addition, the expression cassette may contain selectable markers such as Neo, SV2 Neo, . . .

DETD . . . promoters, such as, for example, the  $\beta$ -galactosidase promoter, trpE promoter, lacZ promoter, T7 promoter, T3 promoter, SP6 promoter, SV40 promoter, **CMV** promoter, and MoMLV LTR.

DETD 4. THE **CMV** H301 GENE

DETD . . . the human immunoglobulin heavy-chain binding protein, and a synthetic sequence of at least 15 bps corresponding in part to the **Kozak** consensus sequence for efficient translational initiation. Although not described in detail here, these signals which affect translation initiation can also. . .

DETD As noted above, the alphavirus cDNA vector construct also includes a 3' sequence which controls transcription **termination**. A representative example of such a sequence is set forth in more detail below in Examples 2 and 3.

DETD . . . vector may be selected to hybridize to any pathogen-specific RNA, for instance target cells infected by pathogens such as HIV, **CMV**, HBV, HPV and HSV.

DETD . . . that any truncation leave intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational **termination** codons may be introduced downstream of the immunogenic region. Insertion of **termination** codons will prematurely terminate protein expression, thus preventing expression of the transforming portion of the protein.

DETD . . . "HCV", respectively), Human Papiloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, HTLV I, HTLV II and Cytomegalovirus ("**CMV**"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which. . .

DETD . . . cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**. Briefly, such eukaryotic layered vector initiation systems provide a two-stage or "layered" mechanism which controls expression of heterologous nucleotide sequences. . . promoter which is capable of initiating the 5' synthesis of RNA from cDNA (e.g., a 5' promoter), a 3' transcription **termination** site, as well as one or more splice sites and/or a polyadenylation site, if desired. Representative promoters suitable for use. . . Mouse polyoma virus promoter ("Py"), rous sarcoma virus ("RSV"), BK virus and JC virus promoters, MMTV promoter, alphavirus junction region, **CMV** promoter, Adenovirus VA1RNA, rRNA promoter, tRNA methionine promoter, CaMV 35S promoter, nopaline synthetase promoter, and the lac promoter. The second. . .

DETD . . . example, vector systems derived from viruses of the following families: Picornaviridae (e.g., poliovirus, rhinovirus, coxsackieviruses), Caliciviridae, Togaviridae (e.g. alphavirus, rubella), **Flaviviridae** (e.g., yellow fever), Coronaviridae (e.g., HCV, TGEV, IBV, MHV, BCoV), Bunyaviridae, Arenaviridae, Retroviridae (e.g., RSV, MoMLV, HIV, HTLV), hepatitis delta. . .

DETD . . . transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**. Within various embodiments, the viral junction region may be modified, such that viral transcription of the subgenomic fragment is merely. . .

DETD . . . auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV HTLV I, HTLV II, **CMV**, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease.

DETD . . . cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**. Within one embodiment, such constructs may be constructed of the following ordered elements: a 5' eukaryotic promoter capable of initiating. . . alphavirus non-structural proteins, a viral junction region, a heterologous sequence, an

**termination/polyadenylation signal sequence.** Such alphavirus cDNA expression vectors may also include intervening sequences (introns), which are spliced from the pre-RNA in.

DETD . . . occurs from the pKSSINBVd1JR construct. Junction region changes at Sindbis nts 7600 and 7602 are downstream of the NSP 4 **termination** codon and upstream of the structural proteins initiation codon.

DETD . . . are observed among the eight alphaviruses sequenced (Semliki Forest virus, Middleburg virus, Ross River virus, O'Nyong Nyong virus, Eastern Equine **Encephalitis virus**, Western Equine **Encephalitis virus**, and Venezuelan Equine **Encephalitis virus**) are given below:

DETD In order to insert the luciferase gene into the pVGELVIS-SINBV vector, the SV40 intron and transcription **termination** sequences at the 3'-end of luciferase must be removed so that when the pre-RNA, transcribed from the plasmid DNA luciferase.

DETD . . . expression, is enhanced by several modifications to the pVGELVIS-SINBV-luc vector. These modifications include alternate RNA polymerase II promoters and transcription **termination** signals, the addition of intron sequences and ribozyme processing signals in the vector construct, and substitution with a smaller plasmid.

DETD The transcription **termination** signals from the SV40 early region or Bovine growth hormone are inserted between the Sac I and Eco RI sites of pBGS131. The SV40 nts between viral nts 2643 to 2563 containing the early region transcription **termination** sequences are isolated by PCR amplification using the primer pair shown below and the pBR322/SV40 plasmid (ATCC # 45019) as.

DETD The Bovine growth hormone transcription **termination** sequences are isolated by PCR amplification using the primer pair shown below and the pCDNA3 plasmid (Invitrogen) as template.

DETD In additional modifications to the ELVIS vector, the transcription **termination** sequences are fused directly to the 3'-end Sindbis sequences, resulting in deletion of the polyadenylate tract; or alternatively the antigenomic. . . ribozyme sequence of hepatitis delta virus (HDV) is inserted between the 3'-polyadenylate tract of the ELVIS vector and the transcription **termination** signals.

DETD In the second vector 3'-end configuration, the SV40 or BGH transcription **termination** signals are fused directly to the 3'-end of the ELVIS vector corresponding to Sindbis nt 11,700 and the polyadenylate tract.

DETD . . . a polyadenylate tract are combined with the plasmid constructions containing the HDV ribozyme sequence and the SV40 or BGH transcription **termination** signals. This construction corresponds to the insertion of pKSSINBV and pKSSINBV-luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids. Alternatively, . . . precisely at the viral 3' end corresponding to viral nt 11,700 are linked directly to the SV40 or BGH transcription **termination** signals. This construction corresponds to the insertion of pKSSINBVd1A and pKSSINBVd1A-luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids.

DETD Using the same overlapping PCR approach, the **CMV** promoter is positioned at the 5' viral end such that transcription initiation results in the addition of a single non-viral nucleotide at the Sindbis 5' end. Amplification of the **CMV** promoter in the first primary PCR reaction is accomplished in a reaction containing the pCDNA3 plasmid and the following primer.

DETD Forward primer: pCBgl233F (buffer sequence/Bgl II recognition sequence/**CMV** promoter nts 1-22):

DETD Reverse primer: SNCMV1142R (SIN nts 8-1/**CMV** pro nts 1142-1108):

DETD Forward primer: CMVSIN1F (**CMV** pro nts 1124-1142/SIN nts 1-20):

DETD Forward primer: pCBgl233F (buffer sequence/Bgl II recognition sequence/**CMV** promoter nts 1-22):

DETD . . . plasmid DNA (FIG. 22A). The data also demonstrate that direct linkage between the Sindbis virus 3'-end and two different transcription **termination/polyadenylation** signals, resulting in deletion of the synthetic A<sub>25</sub> tract, decreased the activity of the DNA vector by more than three.

DETD . . . of in vitro synthesized transcripts, the cDNA is exchanged into a vector containing an RNA polymerase II promoter and transcription **termination/polyadenylation** sequence, as described in Example 2. For plant applications, such promoter and **termination** sequences are chosen from the appropriate plant systems (e.g., CaMV 35S promoter (Guilley et al., Cell 30:763-773, 1982), and nopaline synthase promoter and transcription **termination** sequence (Sanders et al., Nucleic Acids Res. 15:1543-1558). Vector constructs derived from these infectious genomic cDNA clones is subsequently accomplished.

DETD . . . downstream of the disabled junction region in the pKSSINBVd1JR vector, which allows ribosomal scanning in genomic mRNA from non-structural gene **termination** to the heterologous genes. The heterologous proteins are expressed from genomic length mRNA by ribosomal scanning. This extends the life.

DETD . . . et al., Gene 108:167-174, 1991). The sequences surrounding the ATG start codon from bases -9 to +1 conform to the **Kozak** consensus sequence for efficient translational initiation (**Kozak**, Cell

corresponding to the carboxy terminal amino acid is changed to T, . . .

DETD . . . gpt, for the current neomycin resistance, or other promoter elements, for example Drosophila metallothionein or hsp 70, for the current **CMV**, MuLV, and SV40 promoters, may be readily accomplished given the disclosure provided herein.

DETD . . . map, respectively, to the authentic AUG translational start and UGA translational stop sites, including the surrounding nucleotides corresponding to the **Kozak** consensus sequence for efficient translational initiation at Sindbis nt 7638. The forward primer is complementary to Sindbis nts 7638-7661, and. . .

DETD . . . structural protein gene mRNA, the alphavirus structural protein gene sequences, the 3'-end alphavirus sequences required for replication, and a transcription **termination**/polyadenylation sequence. Because of an upstream open-reading frame which ends in translation **termination** codons prior to the AUG start site of the structural protein genes, expression of the alphavirus structural proteins can occur. . .

DETD In addition to their respective complementarities, primer SIN276-SPE contains a flanking UAA translation **termination** codon and SpeI recognition sequence at its 5' end. PCR amplification of the fragment containing Sindbis 5'-end sequences fused to. . .

DETD Modifications of the **CMV** promoter/antisense-Sindbis structural protein vector also can be constructed using other viral, cellular, or insect-based promoters. Using common molecular biology techniques known in the art, the **CMV** promoter can be switched out of the Invitrogen pcDNA3 vector and replaced by promoters such as those listed previously. Other. . . use of longer or shorter HDV or other catalytic ribozyme sequences for transcript processing, the substitution of a precise transcription **termination** signal for the catalytic ribozyme sequence, or the antisense expression of structural protein gene cassettes using any downstream sequence recognized. . .

DETD . . . vector and the Astrovirus expression cassette, between the Astrovirus junction region and structural gene ATG, and between the structural gene **termination** codon and the Astrovirus 3' end. In order to generate wild type virus, this dual recombination event would have to. . .

DETD . . . glycoproteins E1 and/or E2 from other alphaviruses or their variants is performed to alter tissue tropism. For example, Venezuelan equine **encephalitis virus** (VEE) is an alphavirus which exhibits tropism for cells of lymphoid origin, unlike its Sindbis virus counterpart. Therefore, Sindbis-derived vector. . .

DETD . . . is amplified with a primer pair whose 5'-ends map, respectively, to the authentic AUG translational start site, including the surrounding **Kozak** consensus sequence, and UGA translational stop site. The forward primer is complementary to VEE nucleotides 7553-7579, and the reverse primer. . .

DETD . . . Madison, Wis.) by digestion of pcDNA3 with Apa I and EcoRV followed by GENECLAN.TM. purification. The resulting construct, containing a **CMV** promoter which transcribes an antisense reporter cassette RNA of the configuration Sindbis 3'-end sequence/LacZ gene/junction region/Sindbis 5'-end sequence/HDV ribozyme, is. . .

DETD . . . vector. The re-ligated construct contains an antisense LacZ gene and a 3' Sindbis replicase protein recognition sequence downstream from a **CMV** promoter. The resulting construct is known as pcDNAaLacZ-3'Sin. The construct is transfected into BHK cells and utilized as described previously.

DETD . . . assays; (d) map immune response epitopes; and (e) elicit and measure CTL responses to other non-HIV antigens such as mouse **CMV** (MCMV).

DETD Subgenomic mRNA is synthesized in these vectors, which serves as a translational template for the Ad E3 and **CMV** H301 genes. Thus, in these constructions, functional HPV 16 antisense and hairpin ribozyme palliatives will be present on the levels. . .

DETD . . . the E6/E7 or L1 proteins. In these vectors, it would not be desired to include the immunoregulatory Ad E3 or **CMV** H301 genes.

DETD . . . among the group described in Example 5, and the therapeutic palliative. Ordered insertion of the hairpin ribozyme, Ad E3 or **CMV** H301, IRES, and heterologous gene of interest components along the multiple cloning sequence located in the vector between the vector. . .

DETD The coding region and transcriptional **termination** signals of HSV-1 thymidine kinase gene (HSV-TK) are isolated as a 1.8 kb BglII/Pvu II fragment from plasmid 322TK (McKnight. . .

6. The method according to claim 1, further comprising a transcription **termination** sequence.

. . . group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40 promoter, CaMV 35S promoter, nopaline synthetase promoter, and the **CMV** promoter.

. . . from the group consisting of influenza virus, respiratory syncytial virus, IIPV, EBV, HIV, FeLV, FIV, Hantavirus, HTLVI, HTLV, II, and **CMV**.

14 further comprising a transcription **termination** sequence.

. . . group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40 promoter, CaMV 35S promoter, nopaline synthetase promoter, and the **CMV** promoter.

. . . the group consisting of influenza virus, respiratory syncytial virus, HPV, EBV, HIV, FeLV, FIV, Hantavirus, HTLV I, HTLV II, and **CMV**.

L8 ANSWER 10 OF 11 USPATEFULL on STN

1998:91872 Alphavirus structural protein expression cassettes.

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**APPLICATION: US 1996-741881 19961030 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A DNA alphavirus structural protein expression cassette, comprising an inducible promoter and an alphavirus structural protein gene, wherein the promoter directs the expression of the alphavirus structural protein gene upon induction of the promoter within a cell, and wherein prior to induction within the cell, the expression cassette does not express sufficient quantities of structural proteins to be cytotoxic to a BHK cell containing the expression cassette.

2. A DNA alphavirus structural protein expression cassette, comprising a promoter 5' of viral cDNA which initiates the synthesis of RNA from the viral cDNA within a cell, followed by a 5' sequence which initiates transcription of alphavirus RNA from viral RNA, a viral junction region promoter operably-linked to one or more alphavirus structural protein genes and an alphavirus RNA polymerase recognition sequence, with the proviso that the cassette does not direct the expression of all alphavirus nonstructural protein genes.

3. An alphavirus structural protein expression cassette, comprising a promoter and an alphavirus glycoprotein gene, wherein the promoter directs the expression of the alphavirus glycoprotein gene, with the proviso that the promoter does not direct the expression of an alphavirus capsid protein gene.

4. An alphavirus structural protein expression cassette, comprising a promoter and an alphavirus structural protein gene, wherein the promoter directs the expression of the alphavirus structural protein gene, with the proviso that the promoter does not direct the expression of an alphavirus glycoprotein gene, nor the expression of all alphavirus nonstructural protein genes.

5. An alphavirus structural protein expression cassette, comprising a promoter, an alphavirus structural protein gene, and a heterologous ligand sequence, wherein the promoter directs the expression of the alphavirus structural protein gene and the heterologous ligand sequence, with the proviso that the promoter does not direct the expression of all alphavirus nonstructural protein genes.

6. An expression cassette according to claim 1, with the proviso that the promoter does not direct the expression of all alphavirus nonstructural protein genes.

7. An expression cassette according to claim 3, with the proviso that the promoter does not direct the expression of all alphavirus nonstructural protein genes.

8. The expression cassette according to any one of claims 1 to 7 wherein the alphavirus structural protein gene or glycoprotein gene is from a Venezuelan equine **encephalitis virus**.

9. The expression cassette according to any one of claims 1 to 7 wherein the alphavirus structural protein gene or glycoprotein gene is from a Ross River virus.

10. The expression cassette according to any one of claims 1 to 7 wherein the alphavirus structural protein gene or glycoprotein gene is from a Semliki Forest virus.

11. The expression cassette according to any one of claims 1 to 7



from a Sindbis virus.

12. The expression cassette according to any one of claims 1, 2, 4, 5 or 6, wherein the alphavirus structural protein gene encodes an alphavirus capsid protein.

13. The expression cassette of any one of claims 1, 2, 3, 5, 6 or 7, wherein the alphavirus structural protein gene or glycoprotein gene encodes a protein selected from the group consisting of alphavirus structural proteins E3, E2 and E1.

14. The expression cassette according to any one of claims 3, 4, 5, or 7, wherein the cassette is a DNA cassette.

15. The expression cassette according to any one of claims 2, 3, 4, 5, 6, or 7, wherein the promoter is an inducible promoter which directs the expression of a protein within a cell upon induction of the promoter by an inducer.

16. The expression cassette according to claim 14 wherein the promoter is selected from the group consisting of metallothionein, heat shock protein 65, heat shock protein 85, and MMTV.

17. The expression cassette according to claim 14 wherein the promoter is selected from the group consisting of Drosophila actin 5C distal, SV40, Py, RSV, BK, JC, MuLV, **CMV**, and VA1RNA.

18. The expression cassette according to claim 3 or 7, wherein said expression cassette is an RNA expression cassette.

19. The expression cassette according to claim 4 or 5, wherein said expression cassette is an RNA expression cassette.

20. The expression cassette according to claim 18 wherein the promoter is an alphavirus junction region.

21. The expression cassette according to claim 19 wherein the promoter is an alphavirus junction region.

22. A cell containing one or more alphavirus structural protein expression cassettes according to any one of claims 1 to 7.

23. A cell according to claim 22, wherein said cell is a packaging cell, and wherein said cell, upon introduction of an alphavirus vector construct, produces recombinant alphavirus particles.

24. A method of making recombinant alphavirus particles, comprising introducing into a population of cells an alphavirus structural protein expression cassette according to any one of claims 1, 2, 5, 6 or 7, and a vector selected from the group consisting of an alphavirus vector construct, a eukaryotic layered vector initiation system, an RNA vector replicon, and a recombinant vector particle, such that recombinant alphavirus particles are produced.

25. The method according to claim 24, further comprising the step of harvesting recombinant alphavirus particles from said population of cells.

26. A method of making recombinant alphavirus particles, comprising introducing into a population of cells (a) an alphavirus structural protein expression cassette according to claim 3 or 7, (b) a vector selected from the group consisting of an alphavirus vector construct, a eukaryotic layered vector initiation system, an RNA vector replicon, and a recombinant vector particle, and (c) an expression cassette comprising a promoter and an alphavirus capsid gene, wherein the promoter directs the expression of the alphavirus capsid gene, with the proviso that the promoter does not direct the expression of an alphavirus glycoprotein gene, such that recombinant alphavirus particles are produced.

27. The method according to claim 26, further comprising the step of harvesting recombinant alphavirus particles from said population of cells.

28. A method of making recombinant alphavirus particles, comprising introducing into a population of cells (a) an alphavirus structural protein expression cassette according to claim 18, (b) a vector selected from the group consisting of an alphavirus vector construct, a eukaryotic layered vector initiation system, an RNA vector replicon, and a recombinant vector particle, and (c) an expression cassette comprising a promoter and an alphavirus capsid gene, wherein the promoter directs the expression of the alphavirus capsid gene, with the proviso that the promoter does not direct the expression of an alphavirus glycoprotein

29. The method according to claim 28, further comprising the step of harvesting recombinant alphavirus particles from said population of cells.

**AI US 1996-741881 19961030 (8)**

**SUMM** . . . transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**.

**SUMM** . . . transcription of the subgenomic fragment is reduced, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**.

**SUMM** . . . transcription of the subgenomic fragment is reduced, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**.

**SUMM** . . . cell, the vector construct being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**.

**SUMM** . . . the vector construct being capable of expressing a heterologous ribonucleic acid sequence, and a 3' DNA sequence which controls transcription **termination**.

**SUMM** . . . from cDNA is selected from the group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40 promoter, and the **CMV** promoter. Within further embodiments, the eukaryotic layered vector initiation systems further comprise a polyadenylation sequence.

**SUMM** . . . group consisting of influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and **CMV**. Within one preferred embodiment, the heterologous sequence obtained from HPV encodes a protein selected from the group consisting of E5, . . .

**SUMM** . . . are complementary to influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and **CMV** sequences.

**SUMM** . . . 5C distal, SV40, heat shock protein 65, heat shock protein 70, Py, RSV, BK, JC, MuLV, MMTV, alphavirus junction region, **CMV** and VA1RNA.

**DETD** . . . reduce viral transcription of the subgenomic fragment, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**. The vector may also include nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, . . .

**DETD** . . . sequence encoding one or more proteins, said proteins preferably comprising alphavirus structural protein(s). Optionally, the expression cassette may include transcription **termination**, splice recognition, and polyadenylation addition sites. Preferred promoters include the **CMV**, MMTV, MoMLV, and adenovirus VA1RNA promoters. In addition, the expression cassette may contain selectable markers such as Neo, SV2 Neo, . . .

**DETD** . . . promoters, such as, for example, the  $\beta$ -galactosidase promoter, trpE promoter, lacZ promoter, T7 promoter, T3 promoter, SP6 promoter, SV40 promoter, **CMV** promoter, and MoMLV LTR.

**DETD** . . . the human immunoglobulin heavy-chain binding protein, and a synthetic sequence of at least 15 bps corresponding in part to the **Kozak** consensus sequence for efficient translational initiation. Although not described in detail here, these signals which affect translation initiation can also. . .

**DETD** As noted above, the alphavirus cDNA vector construct also includes a 3' sequence which controls transcription **termination**. A representative example of such a sequence is set forth in more detail below in Examples 2 and 3.

**DETD** . . . vector may be selected to hybridize to any pathogen-specific RNA, for instance target cells infected by pathogens such as HIV, **CMV**, HBV, HPV and HSV.

**DETD** . . . that any truncation leave intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational **termination** codons may be introduced downstream of the immunogenic region. Insertion of **termination** codons will prematurely terminate protein expression, thus preventing expression of the transforming portion of the protein.

**DETD** . . . "HCV", respectively), Human Papiloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, HTLV I, HTLV II and Cytomegalovirus ("**CMV**"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which. . .

**DETD** . . . cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**. Briefly, such eukaryotic layered vector initiation systems provide a two-stage or "layered" mechanism which controls expression of heterologous nucleotide sequences. . . . promoter which is capable of initiating the 5' synthesis of RNA from cDNA (e.g., a 5' promoter), a 3' transcription **termination** site, as well as one or more splice sites and/or a polyadenylation site, if desired. Representative promoters suitable for use. . . Mouse polyoma virus promoter ("Py"), rous sarcoma virus ("RSV"), BK virus and JC virus

Adenovirus VA1RNA, rRNA promoter, tRNA methionine promoter, CaMV 35S promoter, nopaline synthetase promoter, and the lac promoter. The second.

DETD . . . example, vector systems derived from viruses of the following families: Picornaviridae (e.g., poliovirus, rhinovirus, coxsackieviruses), Caliciviridae, Togaviridae (e.g. alphavirus, rubella), **Flaviviridae** (e.g., yellow fever), Coronaviridae (e.g., HCV, TGEV, IBV, MHV, BCV), Bunyaviridae, Arenaviridae, Retroviridae (e.g., RSV, MoMLV, HIV, HTLV), hepatitis delta. . .

DETD . . . transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**. Within various embodiments, the viral junction region may be modified, such that viral transcription of the subgenomic fragment is merely. . .

DETD . . . auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV HTLV I, HTLV II, **CMV**, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease.

DETD . . . cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription **termination**. Within one embodiment, such constructs may be constructed of the following ordered elements: a 5' eukaryotic promoter capable of initiating. . . alphavirus non-structural proteins, a viral junction region, a heterologous sequence, an alphavirus RNA polymerase recognition sequence, and a 3' transcription **termination**/polyadenylation signal sequence. Such alphavirus cDNA expression vectors may also include intervening sequences (introns), which are spliced from the pre-RNA in. . .

DETD . . . occurs from the pKSSINBVdlJR construct. Junction region changes at Sindbis nts 7600 and 7602 are downstream of the NSP 4 **termination** codon and upstream of the structural proteins initiation codon.

DETD . . . are observed among the eight alphaviruses sequenced (Semliki Forest virus, Middleburg virus, Ross River virus, O'Nyong Nyong virus, Eastern Equine **Encephalitis virus**, Western Equine **Encephalitis virus**, and Venezuelan Equine **Encephalitis virus**) are given below:

DETD In order to insert the luciferase gene into the pVGELVIS-SINBV vector, the SV40 intron and transcription **termination** sequences at the 3'-end of luciferase must be removed so that when the pre-RNA, transcribed from the plasmid DNA luciferase. . .

DETD . . . expression, is enhanced by several modifications to the pVGELVIS-SINBV-luc vector. These modifications include alternate RNA polymerase II promoters and transcription **termination** signals, the addition of intron sequences and ribozyme processing signals in the vector construct, and substitution with a smaller plasmid. . .

DETD The transcription **termination** signals from the SV40 early region or Bovine growth hormone are inserted between the Sac I and Eco RI sites of pBGS131. The SV40 nts between viral nts 2643 to 2563 containing the early region transcription **termination** sequences are isolated by PCR amplification using the primer pair shown below and the pBR322/SV40 plasmid (ATCC #45019) as template.

DETD The Bovine growth hormone transcription **termination** sequences are isolated by PCR amplification using the primer pair shown below and the pCDNA3 plasmid (Invitrogen) as template.

DETD In additional modifications to the ELVIS vector, the transcription **termination** sequences are fused directly to the 3'-end Sindbis sequences, resulting in deletion of the polyadenylate tract; or alternatively the antigenomic. . . ribozyme sequence of hepatitis delta virus (HDV) is inserted between the 3'-polyadenylate tract of the ELVIS vector and the transcription **termination** signals.

DETD In the second vector 3'-end configuration, the SV40 or BGH transcription **termination** signals are fused directly to the 3'-end of the ELVIS vector corresponding to Sindbis nt 11,700 and the polyadenylate tract. . .

DETD . . . a polyadenylate tract are combined with the plasmid constructions containing the HDV ribozyme sequence and the SV40 or BGH transcription **termination** signals. This construction corresponds to the insertion of pKSSINBV and pKSSINBV-luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids. Alternatively,. . . precisely at the viral 3' end corresponding to viral nt 11,700 are linked directly to the SV40 or BGH transcription **termination** signals. This construction corresponds to the insertion of pKSSINBVdlA and pKSSINBVdlA-luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids.

DETD Using the same overlapping PCR approach, the **CMV** promoter is positioned at the 5' viral end such that transcription initiation results in the addition of a single non-viral nucleotide at the Sindbis 5' end. Amplification of the **CMV** promoter in the first primary PCR reaction is accomplished in a reaction containing the pCDNA3 plasmid and the following primer. . .

DETD Forward primer: pCBgl233F (buffer sequence/Bgl II recognition sequence/**CMV** promoter nts 1-22)

DETD Reverse primer: SNCMV1142R (SIN nts 8-1/**CMV** pro nts 1142-1108)

DETD Forward primer: pCBg1233F (buffer sequence/Bgl II recognition sequence/**CMV** promoter nts 1-22)

DETD . . . plasmid DNA (FIG. 22A). The data also demonstrate that direct linkage between the Sindbis virus 3'-end and two different transcription **termination**/polyadenylation signals, resulting in deletion of the synthetic A<sub>25</sub> tract, decreased the activity of the DNA vector by more than three. . .

DETD . . . of in vitro synthesized transcripts, the cDNA is exchanged into a vector containing an RNA polymerase II promoter and transcription **termination**/polyadenylation sequence, as described in Example 2. For plant applications, such promoter and **termination** sequences are chosen from the appropriate plant systems (e.g., CaMV 35S promoter (Guilley et al., Cell 30:763-773, 1982), and nopaline synthase promoter and transcription **termination** sequence (Sanders et al., Nucleic Acids Res. 15:1543-1558). Vector constructs derived from these infectious genomic cDNA clones is subsequently accomplished. . .

DETD . . . downstream of the disabled junction region in the pKSSINBVdlJR vector, which allows ribosomal scanning in genomic mRNA from non-structural gene **termination** to the heterologous genes. The heterologous proteins are expressed from genomic length mRNA by ribosomal scanning. This extends the life. . .

DETD . . . et al., Gene 108:167-174, 1991). The sequences surrounding the ATG start codon from bases -9 to +1 conform to the **Kozak** consensus sequence for efficient translational initiation (**Kozak**, Cell 44:283-292, 1986). Where possible, the 3' terminal nucleotide corresponding to the carboxy terminal amino acid is changed to T. . .

DETD . . . gpt, for the current neomycin resistance, or other promoter elements, for example Drosophila metallothionein or hsp 70, for the current **CMV**, MuLV, and SV40 promoters, may be readily accomplished given the disclosure provided herein.

DETD . . . map, respectively, to the authentic AUG translational start and UGA translational stop sites, including the surrounding nucleotides corresponding to the **Kozak** consensus sequence for efficient translational initiation at Sindbis nt 7638. The forward primer is complementary to Sindbis nts 7638-7661, and. . .

DETD . . . structural protein gene mRNA, the alphavirus structural protein gene sequences, the 3'-end alphavirus sequences required for replication, and a transcription **termination**/polyadenylation sequence. Because of an upstream open-reading frame which ends in translation **termination** codons prior to the AUG start site of the structural protein genes, expression of the alphavirus structural proteins can occur. . .

DETD In addition to their respective complementarities, primer SIN276-SPE contains a flanking UAA translation **termination** codon and SpeI recognition sequence at its 5' end. PCR amplification of the fragment containing Sindbis 5'-end sequences fused to. . .

DETD Modifications of the **CMV** promoter/antisense-Sindbis structural protein vector also can be constructed using other viral, cellular, or insect-based promoters. Using common molecular biology techniques known in the art, the **CMV** promoter can be switched out of the Invitrogen pcDNA3 vector and replaced by promoters such as those listed previously. Other. . . use of longer or shorter HDV or other catalytic ribozyme sequences for transcript processing, the substitution of a precise transcription **termination** signal for the catalytic ribozyme sequence, or the antisense expression of structural protein gene cassettes using any downstream sequence recognized. . .

DETD . . . vector and the Astrovirus expression cassette, between the Astrovirus junction region and structural gene ATG, and between the structural gene **termination** codon and the Astrovirus 3' end. In order to generate wild type virus, this dual recombination event would have to. . .

DETD . . . glycoproteins E1 and/or E2 from other alphaviruses or their variants is performed to alter tissue tropism. For example, Venezuelan equine **encephalitis virus** (VEE) is an alphavirus which exhibits tropism for cells of lymphoid origin, unlike its Sindbis virus counterpart. Therefore, Sindbis-derived vector. . .

DETD . . . is amplified with a primer pair whose 5'-ends map, respectively, to the authentic AUG translational start site, including the surrounding **Kozak** consensus sequence, and UGA translational stop site. The forward primer is complementary to VEE nucleotides 7553-7579, and the reverse primer. . .

DETD . . . Madison, Wis.) by digestion of pcDNA3 with Apa I and EcoRV followed by GENECLAN.TM. purification. The resulting construct, containing a **CMV** promoter which transcribes an antisense reporter cassette RNA of the configuration Sindbis 3'-end sequence/LacZ gene/junction region/Sindbis 5'-end sequence/HDV ribozyme, is. . .

DETD . . . vector. The re-ligated construct contains an antisense LacZ gene and a 3' Sindbis replicase protein recognition sequence downstream from a **CMV** promoter. The resulting construct is known as pcDNAaLacZ-3'Sin. The construct is transfected into BHK cells and utilized as described previously.

DETD . . . assays; (d) map immune response epitopes; and (e) elicit and

DETD Subgenomic mRNA is synthesized in these vectors, which serves as a translational template for the Ad E3 and **CMV** H301 genes. Thus, in these constructions, functional HPV 16 antisense and hairpin ribozyme palliatives will be present on the levels. . . .

DETD . . . the E6/E7 or L1 proteins. In these vectors, it would not be desired to include the immunoregulatory Ad E3 or **CMV** H301 genes.

DETD . . . among the group described in Example 5, and the therapeutic palliative. Ordered insertion of the hairpin ribozyme, Ad E3 or **CMV** H301, IRES, and heterologous gene of interest components along the multiple cloning sequence located in the vector between the vector. . . .

DETD The coding region and transcriptional **termination** signals of HSV-1 thymidine kinase gene (HSV-TK) are isolated as a 1.8 kb Bgl II/Pvu II fragment from plasmid 322TK. . . .

. . . one of claims 1 to 7 wherein the alphavirus structural protein gene or glycoprotein gene is from a Venezuelan equine **encephalitis virus**.

. . . wherein the promoter is selected from the group consisting of Drosophila actin 5C distal, SV40, Py, RSV, BK, JC, MuLV, **CMV**, and VA1RNA.

L8 ANSWER 11 OF 11 USPATFULL on STN

97:3820 Genetic immunization.

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US 5593972 19970114

**APPLICATION: US 1993-125012 19930921 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A method of immunizing an individual comprising: injecting into skeletal muscle tissue of said individual at a site on said individual's body, bupivacaine and a DNA molecule that comprises a DNA sequence that encodes an antigen from a pathogen, said DNA sequence operatively linked to regulatory sequences which control the expression of said DNA sequence; wherein said DNA molecule is taken up by cells in said skeletal muscle tissue, said DNA sequence is expressed in said cells and an immune response is generated against said antigen.

2. The method of claim 1 wherein said pathogen is an intracellular pathogen.

3. The method of claim 1 wherein said pathogen is a virus selected from the group consisting of: human immunodeficiency virus, HIV; human T cell leukemia virus, HTLV; influenza virus; hepatitis A virus; hepatitis B virus; hepatitis C virus; human papilloma virus, HPV; Herpes simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus, **CMV**; Epstein-Barr virus, EBR; rhinovirus; and, coronavirus.

4. The method of claim 1 wherein said pathogen is HIV and said DNA molecule comprises a DNA sequence that encodes an HIV antigen.

5. The method of claim 1 wherein at least two non-identical DNA molecules are injected into skeletal muscle tissue of said individual at different sites on said individual's body, said bupivacaine being injected into each of the different sites of an individual; said non-identical DNA molecules each comprising DNA sequences encoding one or more pathogen antigens of the same pathogen.

6. A method of immunizing an individual comprising: injecting into skeletal muscle tissue of said individual at a site on said individual's body, bupivacaine and a DNA molecule that comprises a DNA sequence that encodes a hyperproliferative disease-associated protein operatively linked to regulatory sequences; wherein said DNA molecule is taken up by cells in said skeletal muscle tissue, said DNA sequence is expressed in said cells, and an immune response is generated against said hyperproliferative disease-associated protein.

7. The method of claim 6 wherein said DNA molecule comprises a DNA sequence encoding a target protein selected from the group consisting of: protein products of oncogenes myb, myc, fyn, ras, src, neu and trk; protein products of translocation gene bcr/abl; P53; variable regions of antibodies made by B cell lymphomas; and variable regions of T cell receptors of T cell lymphomas.

8. A method of immunizing an individual comprising: injecting into skeletal muscle tissue of said individual, bupivacaine and a DNA

disease-associated protein operatively linked to regulatory sequences; wherein said DNA molecule is taken up by cells in said skeletal muscle tissue, said DNA sequence is expressed in said cells and an immune response is generated against said autoimmune disease-associated protein.

9. The method of claim 8 wherein said DNA molecule comprises a DNA sequence encoding a target protein selected from the group consisting of: variable regions of antibodies involved in B cell mediated autoimmune disease; and variable regions of T cell receptors involved in T cell mediated autoimmune disease.

AI US 1993-125012 19930921 (8)

DETD . . . the individual. Regulatory elements for DNA include a promoter and a polyadenylation signal. In addition, other elements, such as a **Kozak** region, may also be included in the genetic construct.

DETD . . . the DNA or RNA molecule that comprises a nucleotide sequence which encodes the target protein and which includes initiation and **termination** signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of. . .

DETD . . . Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (**CMV**) such as the **CMV** immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as. . .

DETD . . . but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from **CMV**, RSV and EBV.

DETD . . . be operably linked to the nucleotide sequence that encodes the target protein. Accordingly, it is necessary for the initiation and **termination** codons to be in frame with the coding sequence.

DETD . . . and a polyadenylation signal. The promoter may be selected from the group consisting of: HIV LTR, human Actin, human Myosin, **CMV**, RSV, Moloney, MMTV, human Hemoglobin, human muscle creatine and EBV. The enhancer may be selected from the group consisting of: human Actin, human Myosin, **CMV**, RSV, human Hemoglobin, human muscle creatine and EBV. The polyadenylation signal may be selected from the group consisting of: LTR. . .

DETD A region from just upstream of the unique PflMI site to just after the vif **termination** codon was amplified via PCR using primers that introduced a non-conservative amino acid change (glu→val) at amino acid 22 of vpr. . .

DETD . . . HXB2 was amplified via PCR and cloned into the expression vector pCND4/neo (Invitrogen). This plasmid drives envelope production through the **CMV** promoter.

DETD . . . expression is MMTV LTR. The promoter may be deleted and replaced with Actin promoter, myosin promoter, HIV LTR promoter and **CMV** promoter.

DETD . . . Actin no yes no

RA-3 Actin no no yes

RA-4 Actin CME yes yes

RA-5 Actin CME yes no

RA-6 Actin CME no yes

RA-7 **CMV** no yes yes

RA-8 **CMV** no yes no

RA-9 **CMV** no no yes

RA-10 **CMV** CME yes yes

RA-11 **CMV** CME yes no

RA-12 **CMV** CME no yes

RA-13 MMTV no yes yes

RA-14 MMTV no yes no

RA-15 MMTV no no yes

RA-16 MMTV CME yes yes

RA-17 MMTV. . .

DETD . . . to be introduced by vaccination. A region from just upstream of the unique PflMI site to just after the vif **termination** codon was amplified via PCR using primers that introduced a non-conservative amino acid change (glu→val) at amino acid 22 of. . .

DETD . . . The HIV 5'LTR promoter can be deleted and replaced with Moloney virus promoter, MMTV LTR, Actin promoter, myosin promoter and **CMV** promoter.

DETD

Construct	Promoter	poly(A)	Ampr
-----------	----------	---------	------

LA-1	Moloney	HIV 3' LTR	yes
------	---------	------------	-----

LA-2	Moloney	SV40	yes
------	---------	------	-----

LA-3	Moloney	HIV 3' LTR	no
------	---------	------------	----

LA-4	Moloney	SV40	no
------	---------	------	----

LA-5	<b>CMV</b>	HIV 3' LTR	yes
------	------------	------------	-----

LA-6	<b>CMV</b>	SV40	yes
------	------------	------	-----

LA-7	<b>CMV</b>	HIV 3' LTR	no
------	------------	------------	----

LA-9	MMTV	HIV 3' LTR	yes
LA-10	MMTV	SV40	yes
LA-11	MMTV	HIV 3' LTR	no
LA-12	MMTV	SV40	no
LA-13	HIV 5'.		

DETD . . . the thymidine kinase promoter and polyadenylation site. The HIV env coding region is placed under the regulatory control of the **CMV** promoter and SV40 polyadenylation site. The HIV env coding region was obtained as a 2.3 kb PCR fragment from HIV/3B.

DETD . . . the thymidine kinase promoter and polyadenylation site. The HIV gag/pol coding region is placed under the regulatory control of the **CMV** promoter and SV40 polyadenylation site. The HIV gag/pol coding region was obtained from HIV MN, Genbank sequence MI7449, and includes.

DETD . . . the thymidine kinase promoter and polyadenylation site. The HIV gag/pol coding region is placed under the regulatory control of the **CMV** promoter and SV40 polyadenylation site. The HIV gag/pol coding region was obtained from HIV MN, Genbank sequence MI7449, and includes.

DETD Several safety features are included in pGAGPOL.rev. These include use of the **CMV** promoter and a non-retroviral poly(A) site. Furthermore, deletion of the  $\psi$  sequence limits the ability to package viral RNA. In.

DETD Several safety features are included in pGAGPOL.rev. These include use of the **CMV** promoter and a non-retroviral poly(A) site. Furthermore, tat has been deleted and a 50% deletion of nef yields an "inactive".

DETD Step 4a. Digest with BamHI and ligate with the **CMV** promoter obtained by PCR of pCEP4 (Invitrogen, San Diego Calif.) with primers SEQ ID NO.:27 and SEQ ID NO.:28.

DETD . . . be any promoter functional in a human cell. It is preferred that the promoter is an SV40 promoter or a **CMV** promoter, preferably a **CMV** immediate early promoter. The polyadenylation signal may be any polyadenylation signal functional in a human cell. It is preferred that . . . separated by non-coding regions. In order to be properly expressed, a coding region must have an initiation codon and a **termination** codon.

DETD . . . be any promoter functional in a human cell. It is preferred that the promoter is an SV40 promoter or a **CMV** promoter, preferably a immediate early **CMV** promoter. The polyadenylation signal may be any polyadenylation signal functional in a human cell. It is preferred that the polyadenylation . . . separated by non-coding regions. In order to be properly expressed, a coding region must have an initiation codon and a **termination** codon.

DETD . . . identical to or substantially similar to epitopes of HIV proteins. The coding sequence is under the regulatory control of the **CMV** immediate early promoter and the SV40 minor polyadenylation signal.

DETD . . . encodes at least one HIV protein or a fragment thereof. The coding sequence is under the regulatory control of the **CMV** immediate early promoter and the SV40 minor polyadenylation signal. The HIV protein is selected from the group consisting of gag.

DETD . . . identical to or substantially similar to epitopes of HIV proteins. The coding sequence is under the regulatory control of the **CMV** immediate early promoter and the SV40 minor polyadenylation signal. The two expression units are encoded in opposite directions of each.

DETD . . . protein or a fragment thereof. Each expression unit comprises a coding sequence that is under the regulatory control of the **CMV** immediate early promoter and the SV40 minor polyadenylation signal. The HIV protein is selected from the group consisting of gag.

DETD Examples include: (Medical) **dengue**, yellow fever, Japanese encephalitis, St. Louis encephalitis and tick borne encephalitis viruses.

DETD . . . (Medical) these viruses are not placed in a family yet but are believed to be either a togavirus or a **flavivirus**. Most similarity is with togavirus family.

. . . B virus; hepatitis C virus; human papilloma virus, HPV; Herpes simplex 1 virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalovirus, **CMV**; Epstein-Barr virus, EBR; rhinovirus; and, coronavirus.

=> d his

(FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006)

FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

E CHANG G J J/IN  
E CHANG GWONG J J/IN  
L1 2 S E4  
E KONISHI E/AU  
E KONISHI E/IN

L3 3918 S L2 AND KOZAK  
L4 3136 S L3 AND TERMINATION  
L5 0 S L4 AND (POLY W A)  
L6 760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR  
L7 33 S L6 AND CMV/CLM  
L8 11 S L7 AND AY<1999

=> s 16 and pCDNA3?  
7723 PCDNA3?  
L9 498 L6 AND PCDNA3?

=> s 19 and ay<1999  
2811963 AY<1999  
L10 9 L9 AND AY<1999

=> s 110 not 18  
L11 3 L10 NOT L8

=> d 111,cbib,clm,kwic,1-3

L11 ANSWER 1 OF 3 USPATFULL on STN

2002:238871 Recombinant alphavirus-based vectors with reduced inhibition of cellular macromolecular synthesis.

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US 6451592 B1 20020917

**APPLICATION: US 1997-944465 19971006 (8)**

DOCUMENT TYPE: Utility; GRANTED.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. An alphavirus vector construct, comprising: (a) a 5' promoter which directs synthesis of viral RNA in vitro from cDNA, (b) a 5' sequence which directs transcription of alphavirus RNA, (c) a nucleic acid molecule which operably encodes all four alphaviral nonstructural proteins, (d) an alphavirus RNA polymerase recognition sequence, and (e) a 3' polyadenylate tract; wherein a sequence which encodes nonstructural protein 2 (nsP2) is altered such that, when it is operably incorporated into an RNA vector replicon, the time required to reach 50% inhibition of host cell directed macromolecular synthesis following expression in mammalian cells is increased, as compared to RNA vector replicons having a wild-type alphavirus nsP2.

2. The alphavirus vector construct according to claim 1 wherein said 5' promoter which initiates synthesis of viral RNA in vitro from cDNA is a bacteriophage promoter.

3. The alphavirus vector construct according to claim 2 wherein said bacteriophage promoter is an SP6 promoter.

4. The alphavirus vector construct according to claim 2 wherein said bacteriophage promoter is a T7 or T3 promoter.

5. An alphavirus RNA vector replicon capable of translation in a eukaryotic system, comprising: (a) a 5' sequence which initiates transcription of alphavirus RNA, (b) a nucleic acid molecule which operably encodes all four alphaviral nonstructural proteins, (c) an alphavirus junction region promoter (d) an alphavirus RNA polymerase recognition sequence; and (e) a 3' polyadenylate tract; wherein a sequence which encodes nonstructural protein 2 (nsP2) is altered such that, when it is operably incorporated into an RNA vector replicon, the time required to reach 50% inhibition of hostmels directed macromolecular synthesis following expression in mammalian cells is increased, as compared to RNA vector replicons having a wild-type alphavirus nsP2.

6. The alphavirus vector construct according to claim 1 or the alphavirus RNA vector replicon according to claim 5 wherein said sequence encoding said altered nonstructural protein has a mutation within a Leu-Xaa-Pro-Gly-Gly motif SEQ ID NO: 119.

7. The alphavirus vector construct according to claim 1 or the alphavirus RNA vector replicon according to claim 5 wherein said sequence encoding said altered nonstructural protein has a mutation one to three amino acids upstream or downstream of a Leu-Xaa-Pro-Gly-Gly motif SEQ ID NO: 119.



8. The alphavirus vector construct according to claim 1 or the alphavirus RNA vector replicon according to claim 5 wherein said alphavirus is Sindbis virus.
9. The alphavirus vector construct according to claim 1 or the alphavirus RNA vector replicon according to claim 5 wherein said alphavirus is Ockelbo virus.
10. The alphavirus vector construct according to claim 1 or the alphavirus RNA vector replicon according to claim 5 wherein said alphavirus is Semliki Forest virus.
11. The alphavirus vector construct according to claim 1 or the alphavirus RNA vector replicon according to claim 8 wherein said alphavirus is Venezuelan equine **encephalitis virus**.
12. The alphavirus vector construct according to claim 1 or the alphavirus RNA vector replicon according to claim 8 wherein said alphavirus is Ross River virus.
13. The alphavirus vector construct according to claim 1 wherein said alphavirus vector construct further comprises an alphavirus junction region promoter.
14. The alphavirus vector construct according to claim 13 or the alphavirus RNA vector replicon according to claim 5 wherein said alphavirus vector construct or said alphavirus RNA vector replicon further comprises a heterologous nucleic acid sequence.
15. The alphavirus vector construct or RNA vector replicon according to claim 14 wherein said heterologous nucleic acid sequence encodes a lymphokine.
16. The alphavirus vector construct or RNA vector replicon according to claim 15 wherein said lymphokine is interleukin-2 (IL-2).
17. The alphavirus vector construct or RNA vector replicon according to claim 14 wherein said heterologous nucleic acid sequence encodes an interferon.
18. The alphavirus vector construct or RNA vector replicon according to claim 14 wherein said heterologous nucleic acid sequence encodes a growth factor.
19. The alphavirus vector construct or RNA vector replicon according to claim 18 wherein said growth factor is basic Fibroblast Growth Factor (FGF).
20. The alphavirus vector construct or RNA vector replicon according to claim 18 wherein said growth factor is Platelet-derived Growth Factor (PDGF).
21. The alphavirus vector construct or RNA vector replicon according to claim 18 wherein said growth factor is a bone morphogenetic protein.
22. The alphavirus vector construct or RNA vector replicon according to claim 14 wherein said heterologous nucleic acid sequence encodes an antigen from a pathogenic agent.
23. The alphavirus vector construct or RNA vector replicon according to claim 22 wherein said pathogenic agent is a virus.
24. The alphavirus vector construct or RNA vector replicon according to claim 23 wherein said virus is selected from the group consisting of hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV).
25. The alphavirus vector construct according to claim 1 or the alphavirus RNA vector replicon according to claim 5 wherein said alphavirus is Sindbis virus and wherein said altered nsP2 has a mutation at amino acid residue 726.
26. A alphavirus vector construct, comprising: (a) a 5' promoter which directs synthesis of viral RNA in vitro from cDNA; (b) a 5' sequence which directs transcription of alphavirus RNA; (c) a nucleic acid sequence which operably encodes all four alphaviral nonstructural proteins; (d) alphavirus junction region promoter sequence; (e) heterologous nucleic acid sequence; (f) an alphavirus RNA polymerase recognition sequence; and (g) a 3' polyadenylate tract; wherein said nucleic acid sequence which operably encodes all four alphaviral nonstructural proteins further comprises an altered nucleic acid sequence encoding for nonstructural protein 2 (nsP2) having a mutation

sequence is operably incorporated into an RNA vector replicon, the time required to reach 50% inhibition of host-cell directed macromolecular synthesis following expression in mammalian cells is increased, as compared to an RNA vector replicon having a wild-type alphavirus nsP2.

**AI US 1997-944465 19971006 (8)**

**SUMM** . . . USA 89:2679-2683, 1992; Dubensky et al., J. Virol. 70:508-519, 1996), Semliki Forest virus (Liljestrom, Bio/Technology 9:1356-1361, 1991), and Venezuelan Equine **Encephalitis virus** (Davis et al., J. Cell. Biochem. Suppl. 19A:10, 1995). In addition, one group has suggested using alphavirus-derived vectors for the . . .

**SUMM** . . . Such vectors may be constructed from a wide variety of alphaviruses (e.g., Semliki Forest virus, Ross River virus, Venezuelan equine **encephalitis virus** or Sindbis virus), and designed to express numerous heterologous sequences (e.g., a sequence corresponding to protein, a sequence corresponding to. . .

**SUMM** . . . one of the above-described nucleic acid molecules. Within one embodiment, the expression vector further comprises a polyadenylation sequence or transcription **termination** sequence 3' to the nucleic acid molecule.

**SUMM** . . . proteins, a selectable marker operably linked to transcription of the expression cassette, and optionally, a 3' sequence which controls transcription **termination**. Within one embodiment, such expression cassettes further comprise a 5' sequence which initiates transcription of alphavirus RNA, a viral junction. . .

**SUMM** . . . promoters, RNA polymerase III promoters, the HSV-TK promoter, RSV promoter, tetracycline inducible promoter, MoMLV promoter, a SV40 promoter and a **CMV** promoter. Within preferred embodiments, the 5' promoter is an inducible promoter as described herein.

**SUMM** . . . export from the nucleus. Within further embodiments, the eukaryotic layered vector initiation systems provided herein may further comprise a transcription **termination** signal.

**DETD** . . . or more biologically active alphavirus structural proteins (e.g., C, E3, E2, 6K, E1), and a 3' sequence which controls transcription **termination**. The expression cassette also may include a 5' sequence which is capable of initiating transcription of an alphavirus RNA (also. . .

**DETD** . . . 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA, a 3' sequence which controls transcription **termination**, splice recognition sequences, a catalytic ribozyme processing sequence, a sequence encoding a selectable marker, and a nuclear export signal.

**DETD** . . . initiation system may also contain splice recognition sequences, a catalytic ribozyme processing sequence, a nuclear export signal, and a transcription **termination** sequence. In certain embodiments, in vivo synthesis of the vector nucleic acid sequence from cDNA may be regulated by the. . .

**DETD** . . . is capable of initiating in vivo the synthesis of viral RNA from cDNA, and a 3' sequence which controls transcription **termination**. Within certain embodiments, the vector construct may further comprise a viral subgenomic "junction region" promoter which may, in certain embodiments,. . .

**DETD** . . . alphavirus stocks. These plasmids include: for Semliki Forest Virus, pSP6-SFV4 (Liljestrom et al., J. Virol. 65:4107-4113, 1991); for Venezuelan equine **encephalitis virus**, pV2000 (Davis et al., Vir. 183:20-31, 1991); for Ross River virus, pRR64 (Kuhn et al., Vir. 182:430-441, 1991). Briefly, for. . .

**DETD** . . . Schlesinger, Trends Biotechnol. 11:18-22, 1993; Dubensky et al., *ibid*), Semliki Forest virus (Liljestrom and Garoff, Bio/Technology 9:1356-1361, 1991), Venezuelan equine **encephalitis virus** (Davis et al., J. Cell. Biochem. Suppl. 19A:310, 1995), poliovirus (Choi et al., J. Virol. 65:2875-2883, 1991; Ansardi et al., . . .

**DETD** . . . example, the bacterial  $\beta$ -galactosidase and trpE promoters, and the eukaryotic viral simian virus 40 (SV40) (e.g., early or late), cytomegalovirus (**CMV**) (e.g., immediate early), Moloney murine leukemia virus (MoMLV) or Rous sarcoma virus (RSV) LTR, and herpes simplex virus (HSV) (thymidine. . .

**DETD** . . . as one of two large polyproteins, known as P123 or P1234, respectively, depending upon (i) whether there is an opal **termination** codon between the coding regions of nsP3 and nsP4 and (ii) if there is such an opal codon present, whether there is translation **termination** of the nascent polypeptide at that point or readthrough and hence production of P1234. The opal **termination** codon is present at the nsP3/nsP4 junction of the alphaviruses SIN (strain AR339 and the SIN-1 strain described herein), AURA, . . . and RR, and thus the P123 and P1234 species are expressed in cells infected with these viruses. In contrast, no **termination** codon is present at the nsP3/nsP4 junction of the alphaviruses SIN (strain AR86, SF, and ONN), and thus only the. . .

. . . Translational readthrough generally occurs about 10%-20% of the time in cells infected with wild type Sindbis virus containing the opal **termination** codon at the nsP3/nsP4 junction. Processing of P123 and P1234 is by a proteinase activity encoded by the one of. . .

heavily phosphorylated state. In alphaviruses whose genomes contain an opal **termination** codon between the nsP3/nsP4 junction, two different proteins are produced depending upon whether or not there is readthrough of the opal **termination** signal. Readthrough results in an nsP3 protein which contains 7 additional carboxy terminal amino acids after cleavage of the polyprotein.. . .

DETD . . . cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence that controls transcription **termination** (e.g., a polyadenylate tract). Such eukaryotic layered vector initiation systems provide a two-stage or "layered" mechanism that controls expression of. . . synthesis of RNA from cDNA (e.g., a 5' eukaryotic promoter), and may further comprise other elements, including a 3' transcription **termination**/polyadenylation site, one or more splice sites, as well as other RNA nuclear export elements, including, for example, the hepatitis B. . . herpes simplex virus (HSV) promoter, BK virus and JC virus promoters, mouse mammary tumor virus (MMTV) promoter, alphavirus junction region, **CMV** promoter, Adenovirus E1 or VAIRNA promoters, rRNA promoters, tRNA methionine promoter, CaMV 35S promoter, nopaline synthetase promoter, tetracycline responsive promoter,. . .

DETD . . . example, vector systems derived from viruses of the following families: Picomaviridae (e.g., poliovirus, rhinovirus, coxsackieviruses), Caliciviridae, Togaviridae (e.g., alphavirus, rubella), **Flaviviridae** (e.g., yellow fever, HCV), Coronaviridae (e.g., HCV, TGEV, IBV, MHV, BCV), Bunyaviridae, Arenaviridae, Retroviridae (e.g., RSV, MoMLV, HIV, HTLV), hepatitis. . .

DETD . . . alphavirus particle, results in the desired phenotype), an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription **termination**/polyadenylation. In addition, a viral junction region which is operably linked to a heterologous sequence to be expressed may be included.. . .

DETD . . . RNA by binding specifically to a tetracycline operator sequence (tetO) located immediately adjacent to a minimal "core" promoter (for example, **CMV**). The binding and transactivation event is reversibly blocked by the presence of tetracycline, and may be "turned on" by removing. . .

DETD . . . of togaviruses, including, but not limited to, alphaviruses (such as Sindbis Virus (e.g., SIN-1 or wild-type Sindbis virus), Venezuelan Equine **Encephalitis virus**, Ross River virus, Eastern Equine **Encephalitis virus**, Western Equine **Encephalitis virus**, and rubiviruses (e.g., rubella).

DETD . . . that any truncation leave intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational **termination** codons may be introduced downstream of the immunogenic region. Insertion of **termination** codons will prematurely terminate protein expression, thus preventing expression of the transforming portion of the protein.

DETD . . . Syncytial Virus, Human Papilloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, HTLV I, HTLV II and Cytomegalovirus ("**CMV**"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which. . .

DETD . . . or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV, HCV, HTLV I, HTLV II, **CMV**, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease. More specifically, within one aspect of. . .

DETD The SIN-1 specific nucleotide sequences of the pRSIN-1g clone was determined by the dideoxy-chain **termination** method. Sequence comparison of 8,000 bp of viral sequence revealed multiple differences between the SIN-1 clone described herein and the. . .

DETD . . . directly. The gene encoding neomycin (G418) resistance was isolated by standard three-cycle PCR amplification, with 1.5 minutes extension, from plasmid **pcDNA3** (Invitrogen, San Diego, Calif.), using the following oligonucleotide primers that were designed to contain flanking Xho I and Not I. . .

DETD . . . phenotype. The gene encoding neomycin (G418) resistance was isolated by standard three-cycle PCR amplification, with 1.5 minutes extension, from plasmid **pcDNA3** (Invitrogen, San Diego, Calif.), using the following oligonucleotide primers that were designed to contain flanking BamH I restriction sites:

DETD The bovine growth hormone (BGH) transcription **termination**/polyadenylation signal was inserted between the Sac I and Eco RI sites of pBGS131 dlXho I. The BGH transcription **termination** sequences were isolated by PCR amplification using the primer pair shown below and the **pcDNA3** plasmid (Invitrogen, San Diego, Calif.) as template.

DETD Forward Primer BGHTTF (buffer sequence/Sac I site/**pcDNA3** nts 1132-1161): (SEQ. ID NO. 37)

DETD Reverse Primer BGHTTR (buffer sequence/Eco RI site/**pcDNA3** nts 1180-1154): (SEQ. ID NO. 38)

DETD . . . of the Sindbis virus expression vector from plasmid

transcription **termination** sequence on the pBGS131 dIXho I plasmid.

DETD Assembly of the Sindbis virus plasmid DNA vector was completed by insertion of the **CMV** promoter juxtaposed with the first 2289 nts of the Sindbis virus genome (includes the 5' viral end and a portion of the nsPs genes) into the pBG/SIN-1BglLF plasmid. Using an overlapping PCR approach, the **CMV** promoter was positioned at the 5' viral end such that transcription initiation results in the addition of a single non-viral nucleotide at the 5' end of the Sindbis virus vector replicon RNA. The **CMV** promoter was amplified in a first PCR reaction from **pCDNA3** (Invitrogen, San Diego, Calif.) using the following primer pair:

DETD Forward Primer: pCBgl233F (buffer sequence/Bgl II recognition sequence/**CMV** promoter nts 1-22): (SEQ. ID NO. 41)

DETD Reverse Primer: SNCMV1142R (SIN nts 8-1/**CMV** pro nts 1142-1108): (SEQ. ID NO. 42)

DETD Forward Primer: CMVSIN1F (**CMV** pro nts 1124-1142/SIN nts 1-20): (SEQ. ID NO. 43)

DETD . . .

Plasmid Transfected RLU at 48 hpt

pBG/SIN-1 ELVS 1.5-SEAP  $18 \pm 1.7$   
pBG/wt ELVS 1.5-SEAP  $94 \pm 10.7$   
**pCDNA3**  $0.13 \pm 0.04$

DETD . . .  $10^9$   
pBG/wt ELVS 1.5-luc  $4.1 \times 10^9$   
pBG/SIN-1 ELVS 1.5-luc  $72.18 \times 10^9$   
pBG/wt ELVS 1.5-luc  $5.8 \times 10^9$   
**pCDNA3** 48 482

DETD . . .  $2232585 \pm 299166$   
pBG/wt ELVS 1.5- $\beta$ -gal  $3514262 \pm 548225$

pBG/SIN-1 ELVS 1.5- $\beta$ -gal  $1203200910 \pm 128036$   
pBG/wt ELVS 1.5- $\beta$ -gal  $1986537 \pm 166869$

**pCDNA3** 3637

DETD . . . were determined. The conventional plasmid vector was constructed by insertion of the lac Z gene (Promega, Madison, Wis.) into the **CMV** promoter-driven pUC-derived expression plasmid multiple cloning site (Invitrogen, San Diego, Calif.), and is known as pCMV- $\beta$ -gal. The results of this. . .

DETD . . . expression cassette components can also be performed. For example, substitution of the MoMLV RNA polymerase II promoter with the stronger **CMV** immediate early (IE) promoter significantly enhances the level of heterologous gene expression in transfected cells (Dubensky et al., J. Virol. 70:508-519, 1996, and Dubensky et al., W/O 95/07994). Further, juxtaposition of introns, for example SV40 small t antigen or **CMV** intron A, either upstream or downstream from the heterologous gene, can increase the level of heterologous gene expression in some. . .

DETD For example, an alphavirus structural protein expression cassette was constructed, whereby primary transcription from a **CMV** immediate early promoter produces an RNA molecule capable of efficient cytoplasmic amplification and structural protein expression only after translation of. . . ribozyme sequence, an additional plasmid from Dubensky et al., (ibid), pDLTRSINGHDV, was used as starting material to reconstruct the modified **CMV**-based DH construct. Plasmid pDLTRSINGHDV, an LTR-based Sindbis genomic clone containing the HDV ribozyme, was digested with Bgl II to remove. . . and purified from a 0.7% agarose gel using GENECLEAN II.TM. (Biol01, San Diego, Calif.). The corresponding 5'-end fragment with a **CMV** promoter was obtained by Bgl II digestion of the Sindbis genomic clone pDCMVSING (Dubensky et al., ibid) and purification from. . . agarose gel using GENECLEAN II, and then ligated into the Bgl II-deleted pDLTRSINGHDV vector to generate the construct pDCMVSINGHDV. This **CMV**-based genomic plasmid with an HDV ribozyme was shown to produce infectious Sindbis virus and cytopathic effect within 24 hr after. . .

DETD Packaging activity of the **CMV**-based DH construct was also highly efficient in non-mammalian cells, for example, C6/36 mosquito cells. The use of such a non-mammalian. . . that both a DNA-based luciferase reporter vector and DH helper vector expressing Sindbis structural proteins, under the control of the **CMV** promoter, were fully functional in C6/36 cells, as demonstrated by luciferase vector packaging.

DETD . . . DNA fragment comprising the 3'-end of Sindbis virus, a synthetic A<sub>40</sub> tract, the antigenomic HDV ribozyme, and a BGH transcription **termination** signal, was removed from plasmid pBG/SIN-1 ELVS 1.5 (Example 5) by digestion with Not I and EcoR I, and purification. . . with alkaline phosphatase, and purified with GENECLEAN II for insertion of remaining 5'-end and nonstructural gene sequences, along with a **CMV IE** promoter. The remaining sequences were obtained by digestion of plasmidpDCMVSING (Dubensky et al., ibid) with Bgl II, purification of the fragment from a 1% agarose gel using GENECLEAN II, and ligation with the linear pBGSV3'BF vector, to create

of this construct for initiation of the Sindbis virus replication cycle was determined by Lipofectamine-mediated.

DETD . . . codons of the nsP1 open reading frame. Briefly, the neomycin resistance gene was amplified by standard three-cycle PCR from the **pCDNA3** vector (Invitrogen, San Diego, Calif.), using the following oligonucleotide primers that were designed to contain flanking BspE I and BamH.

DETD . . . FIG. 14. The configuration of pBGSMCMVdlneo includes, as part of the structural protein expression cassette and controlled by the same **CMV** promoter, a fusion protein comprising the initiator methionine and amino-terminal 121 amino acids of nsP1 and the neomycin resistance gene.

DETD Next, the transcription **termination** signal from the SV40 early region is inserted between the Sac I and Eco RI sites of pBGSMNsp, immediately downstream of the Sindbis sequence. The SV40 viral nucleotides 2643 to 2563, containing the early region transcription **termination** sequences, are isolated by PCR amplification using the primer pair shown below and the pBR322/SV40 plasmid (ATCC #45019), as template.

DETD . . . #2, the neo resistance marker is amplified by standard three cycle PCR with a 1.5 minute extension, from template plasmid **pCDNA3** (Invitrogen, San Diego, Calif.), using the following oligonucleotide primers that are designed to also contain a flanking Nsi I site.

DETD . . . the following oligonucleotide primers that are designed to contain a flanking Xho I site and translation initiation codon in good **Kozak** context, or a flanking Not I site and the translation stop codon.

DETD . . . or no inhibition of host macromolecular synthesis, plus, structural proteins which redirect the tropism of the recombinant particle. Venezuelan equine **encephalitis virus** (VEE) is an alphavirus which exhibits tropism for cells of lymphoid origin, unlike its Sindbis virus counterpart. Therefore, Sindbis-derived vector.

DETD . . . by PCR with a primer pair whose 5'-ends map, respectively, to the authentic AUG translational start site, including the surrounding **Kozak** consensus sequence, and the UGA translational stop site. The forward primer is complementary to VEE nucleotides 7553-7579, and the reverse.

DETD . . . RNA by binding specifically to a tetracycline operator sequence (tetO) located immediately adjacent to a minimal "core" promoter (for example, **CMV**). The binding and transactivation event is reversibly blocked by the presence of tetracycline, and may be "turned on" by removing.

DETD . . . tetracycline-regulated DNA-based alphavirus vector is demonstrated by constructing a modified SIN-1-derived luciferase plasmid vector, which is driven by a tetracycline operator/**CMV** minimal promoter. Using plasmids pBG/SIN-1 ELVS1.5-luc (Example 4) and pBGSV3' (Example 6) as starting material, an approximately 7200 bp fragment, . . .

. . . II. The resulting construct is designated pBGSVd1B/SIN1-luc. Insertion of the remaining sequences, which include the heptamerized tetracycline operator and minimal **CMV** promoter (tetO/**CMV**) linked to Sindbis nucleotides 1-2289, such that transcription will initiate with one additional nonviral nucleotide 5' of Sindbis nucleotide 1, is accomplished by overlapping PCR. In PCR reaction #1, the approximately 370 bp tetO/**CMV** portion of the sequence is amplified by standard three-cycle PCR with a 30 second extension from template plasmid pUHC13-3 (Gossen).

DETD Reverse Primer: 3'**CMV**pro/SINR (5'-Sindbis nts./**CMV** nts.) (SEQ. ID. NO. 89)

DETD . . . from template plasmid pKRSIN-1 (Example 1), using the following oligonucleotide primers that are designed to also contain sequences overlapping the **CMV** promoter nucleotides on one primer.

DETD Forward Primer: CMVSIN5'endF (5'-**CMV** nts./Sindbis nts.) (SEQ. ID. NO. 90)

DETD . . . identified by restriction analysis. This configuration positions the promoter and gpt gene immediately adjacent to a bovine growth hormone transcription **termination** signal. The resulting gpt expression construct is designated pBGSI31 dLXhoI-gpt. Next the entire expression cassette is amplified from plasmid pBGSI31.

. . . according to claim 1 or the alphavirus RNA vector replicon according to claim 8 wherein said alphavirus is Venezuelan equine **encephalitis virus**.

L11 ANSWER 2 OF 3 USPATFULL on STN

2000:18426 Chimeric hepatitis B/hepatitis C virus vaccine.

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US 6025341 20000215

**APPLICATION: US 1997-854531 19970512 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

1. A recombinant DNA molecule comprising a nucleotide coding sequence that encodes a fusion protein, wherein said fusion protein consists of a hepatitis B virus S gene product linked to amino acids 1-69 of the hepatitis C virus core protein.
2. The recombinant nucleic acid molecule of claim 1 wherein the N terminal amino acid of said hepatitis C virus core protein is linked to the C terminal amino acid of said hepatitis B virus S gene product.
3. A method of inducing an immune response against hepatitis C virus in an individual uninfected by hepatitis C virus comprising the step of: administering to said individual the DNA molecule of claim 1 in an amount effective to induce an immune response against hepatitis C virus.
4. A method of treating an individual who is infected with the hepatitis C virus comprising the step of: administering to said individual the DNA molecule of claim 1 in an amount effective to induce an immune response against hepatitis C virus, wherein antibodies are produced.
5. A recombinant DNA molecule comprising a nucleotide sequence that encodes a fusion protein, wherein said fusion protein consists of a hepatitis B virus S gene product linked to amino acids 1-70 to 1-154 of the hepatitis C virus core protein.
6. The recombinant nucleic acid molecule of claim 5 wherein the N terminal amino acid of said hepatitis C virus core protein is linked to the C terminal amino acid of said hepatitis B virus S gene product.
7. A method of inducing an immune response against hepatitis C virus in an individual uninfected by hepatitis C virus comprising the step of: administering to said individual the DNA molecule of claim 5 in an amount effective to induce an immune response against hepatitis C virus.
8. A method of treating an individual who is infected with hepatitis C virus comprising the step of: administering to said individual the DNA molecule of claim 5 in an amount effective to induce an immune response against hepatitis C virus, wherein antibodies are produced.
9. A recombinant DNA molecule comprising a nucleotide sequence that encodes a fusion protein, wherein said fusion protein consists of a fragment of the hepatitis B virus pre S2 gene product linked to the hepatitis B virus S gene product linked to amino acids 1-69 of the hepatitis C virus core protein.
10. The recombinant nucleic acid molecule of claim 9 wherein the N terminal amino acid of said hepatitis B virus S gene product is linked to the C terminal amino acid of said hepatitis B virus pre S2 gene product, and the N terminal amino acid of said hepatitis C virus core protein is linked to the C terminal amino acid of said hepatitis B virus S gene product.
11. A method of inducing an immune response against hepatitis C virus in an individual uninfected by hepatitis C virus comprising the step of: administering to said individual the DNA molecule of claim 9 in an amount effective to induce an immune response against hepatitis C virus.
12. A method of treating an individual who is infected with hepatitis C virus comprising the step of: administering to said individual the DNA molecule of claim 9 in an amount effective to induce an immune response against hepatitis C virus, wherein antibodies are produced.
13. A recombinant DNA molecule comprising a nucleotide sequence that encodes a fusion protein, wherein said fusion protein consists of a fragment of the hepatitis B virus pre S2 gene product linked to the hepatitis B virus S gene product linked to amino acids 1-70 to 1-154 of the hepatitis C virus core protein.
14. The recombinant nucleic acid molecule of claim 13 wherein the N terminal amino acid of said hepatitis B virus S gene product is linked to the C terminal amino acid of said hepatitis B virus pre S2 gene product, and the N terminal amino acid of said hepatitis C virus core protein is linked to the C terminal amino acid of said hepatitis B virus S gene product.
15. A method of inducing an immune response against hepatitis C virus in an individual uninfected by hepatitis C virus comprising the step of: administering to said individual the DNA molecule of claim 13 in an amount effective to induce an immune response against hepatitis C virus.
16. A method of treating an individual who is infected with hepatitis C virus comprising the step of: administering to said individual the DNA molecule of claim 13 in an amount effective to induce an immune response

17. A recombinant DNA molecule comprising a nucleotide sequence that encodes a fusion protein, wherein said fusion protein consists of the hepatitis B virus pre S2 gene product linked to the hepatitis B virus S gene product linked to amino acids 1-69 of the hepatitis C virus core protein.

18. The recombinant nucleic acid molecule of claim 17 wherein the N terminal amino acid of said hepatitis B virus S gene product is linked to the C terminal amino acid of said hepatitis B virus pre S2 gene product, and the N terminal amino acid of said hepatitis C virus core protein is linked to the C terminal amino acid of said hepatitis B virus S gene product.

19. A method of immunizing an individual susceptible to or infected by hepatitis C virus for producing antibodies comprising the step of: administering to said individual the DNA molecule of claim 17 in an amount effective to induce an immune response, wherein antibodies are produced.

20. A method of treating an individual who is infected with hepatitis C virus comprising the step of: administering to said individual the DNA molecule of claim 17 in an amount effective to induce an immune response against hepatitis C virus, wherein antibodies are produced.

21. A recombinant DNA molecule comprising a nucleotide sequence that encodes a fusion protein, wherein said fusion protein consists of the hepatitis B virus pre S2 gene product linked to the hepatitis B virus S gene product linked to amino acids 1-70 to 1-154 of the hepatitis C virus core protein.

22. The recombinant nucleic acid molecule of claim 21 wherein the N terminal amino acid of said hepatitis B virus S gene product is linked to the C terminal amino acid of said hepatitis B virus pre S2 gene product, and the N terminal amino acid of said hepatitis C virus core protein is linked to the C terminal amino acid of said hepatitis B virus S gene product.

23. A method of immunizing an individual susceptible to or infected by hepatitis C virus for producing antibodies comprising the step of: administering to said individual the DNA molecule of claim 21 in an amount effective to induce an immune response, wherein antibodies are produced.

24. A method of treating an individual who is infected with hepatitis C virus comprising the step of: administering to said individual the DNA molecule of claim 21 in an amount effective to induce an immune response against hepatitis C virus, wherein antibodies are produced.

25. The recombinant DNA molecule of any one of claims 1, 5, 9, 13, 17, or 21 comprising a cytomegalovirus promoter, a Rous Sarcoma Virus enhancer, and a polyadenylation sequence, wherein the nucleotide coding sequence is operatively linked to said promoter, enhancer, and polyadenylation sequence.

26. The recombinant DNA molecule of claim 25 further comprising the 5' UTR of hepatitis C virus, wherein said nucleotide coding sequence is operatively linked thereto.

27. A recombinant DNA molecule comprising a nucleotide coding sequence that encodes a fusion protein, wherein said fusion protein is selected from the group consisting of: a fusion protein that consists of the hepatitis B virus S gene product linked to a truncated hepatitis C virus core protein, a fusion protein that consists of a fragment of the the hepatitis B virus pre S2 gene product linked to the hepatitis B virus S gene product linked to a truncated hepatitis C virus core protein, and a fusion protein that consists of the hepatitis B virus pre S2 gene product linked to the hepatitis B virus S gene product linked to a truncated hepatitis C virus core protein.

28. The recombinant DNA molecule of claim 27 wherein said fusion protein consists of the hepatitis B virus S gene product linked to a truncated hepatitis C virus core protein.

29. The recombinant DNA molecule of claim 27 wherein said fusion protein consists of a fragment of the hepatitis B virus pre S2 gene product linked to the hepatitis B virus S gene product linked to a truncated hepatitis C virus core protein.

30. The recombinant DNA molecule of claim 27 wherein said fusion protein consists of the hepatitis B virus pre S2 gene product linked to the hepatitis B virus S gene product linked to a truncated hepatitis C virus

31. A pharmaceutical composition comprising: a) a recombinant DNA molecule of claim 1; wherein said nucleotide sequence is operably linked to regulatory elements functional in human cells; and b) a pharmaceutically acceptable carrier or diluent.

32. The pharmaceutical composition of claim 31 wherein said regulatory elements functional in human cells comprise a cytomegalovirus promoter, a Rous Sarcoma Virus enhancer, a polyadenylation sequence.

33. The pharmaceutical composition of claim 32 further comprising the 5' UTR of hepatitis C virus.

34. A method of immunizing an individual susceptible to or infected by hepatitis C virus for producing antibodies comprising the step of: administering to said individual the pharmaceutical composition of claim 31 in an amount effective to induce an immune response, wherein antibodies are produced.

35. The method of claim 34 wherein bupivacaine is administered to said individual at the site of administration of the pharmaceutical composition.

36. A pharmaceutical composition comprising: a) the recombinant DNA molecule of claim 5, wherein said nucleotide sequence is operably linked to regulatory elements functional in human cells; and b) a pharmaceutically acceptable carrier or diluent.

37. The pharmaceutical composition of claim 36 wherein said regulatory elements functional in human cells comprise a cytomegalovirus promoter, a Rous Sarcoma Virus enhancer, a polyadenylation sequence.

38. The pharmaceutical composition of claim 37 further comprising the 5' UTR of hepatitis C virus.

39. A method of immunizing an individual susceptible to or infected by hepatitis C virus for producing antibodies comprising the step of: administering to said individual the pharmaceutical composition of claim 36 in an amount effective to induce an immune response, wherein antibodies are produced.

40. The method of claim 39 wherein bupivacaine is administered to said individual at the site of administration of the pharmaceutical composition.

41. A pharmaceutical composition comprising: a) the recombinant DNA molecule of claim 9, wherein said nucleotide sequence is operably linked to regulatory elements functional in human cells; and b) a pharmaceutically acceptable carrier or diluent.

42. The pharmaceutical composition of claim 41 wherein said regulatory elements functional in human cells comprise a cytomegalovirus promoter, a Rous Sarcoma Virus enhancer, a polyadenylation sequence.

43. The pharmaceutical composition of claim 42 further comprising the 5' UTR of hepatitis C virus.

44. A method of immunizing an individual susceptible to or infected by hepatitis C virus for producing antibodies comprising the step of: administering to said individual the pharmaceutical composition of claim 41 in an amount effective to induce an immune response, wherein antibodies are produced.

45. The method of claim 44 wherein bupivacaine is administered to said individual at the site of administration of the pharmaceutical composition.

46. A pharmaceutical composition comprising: a) the recombinant DNA molecule of claim 13, wherein said nucleotide sequence is operably linked to regulatory elements functional in human cells; and b) a pharmaceutically acceptable carrier or diluent.

47. The pharmaceutical composition of claim 46 wherein said regulatory elements functional in human cells comprise a cytomegalovirus promoter, a Rous Sarcoma Virus enhancer, a polyadenylation sequence.

48. The pharmaceutical composition of claim 47 further comprising the 5' UTR of hepatitis C virus.

49. A method of immunizing an individual susceptible to or infected by hepatitis C virus for producing antibodies comprising the step of: administering to said individual the pharmaceutical composition of claim



antibodies are produced.

50. The method of claim 49 wherein bupivacaine is administered to said individual at the site of administration of the pharmaceutical composition.

51. A pharmaceutical composition comprising: a) the recombinant DNA molecule of claim 17, wherein said nucleotide sequence is operably linked to regulatory elements functional in human cells; and b) a pharmaceutically acceptable carrier or diluent.

52. The pharmaceutical composition of claim 51 wherein said regulatory elements functional in human cells comprise a cytomegalovirus promoter, a Rous Sarcoma Virus enhancer, a polyadenylation sequence.

53. The pharmaceutical composition of claim 52 further comprising the 5' UTR of hepatitis C virus.

54. A method of immunizing an individual susceptible to or infected by hepatitis C virus for producing antibodies comprising the step of: administering to said individual the pharmaceutical composition of claim 51 in an amount effective to induce an immune response, wherein antibodies are produced.

55. The method of claim 54 wherein bupivacaine is administered to said individual at the site of administration of the pharmaceutical composition.

56. A pharmaceutical composition comprising: a) the recombinant DNA molecule of claim 21, wherein said nucleotide sequence is operably linked to regulatory elements functional in human cells; and b) a pharmaceutically acceptable carrier or diluent.

57. The pharmaceutical composition of claim 56 wherein said regulatory elements functional in human cells comprise a cytomegalovirus promoter, a Rous Sarcoma Virus enhancer, a polyadenylation sequence.

58. The pharmaceutical composition of claim 57 further comprising the 5' UTR of hepatitis C virus.

59. A method of immunizing an individual susceptible to or infected by hepatitis C virus for producing antibodies comprising the step of: administering to said individual the pharmaceutical composition of claim 56 in an amount effective to induce an immune response, wherein antibodies are produced.

60. The method of claim 59 wherein bupivacaine is administered to said individual at the site of administration of the pharmaceutical composition.

**AI** **US 1997-854531** **19970512 (8)**  
**SUMM** . . . positive stranded RNA virus, approximately 9,500 nucleotides in length, which has recently been classified as a separate genus within the **Flavivirus** family (Heinz, F. X., Arch. Virol. (Suppl.), 1992, 4, 163-171). Different isolates show considerable nucleotide sequence diversity leading to the . . .  
**DETD** . . . 69 amino acids of the HCV core protein, and the entire HBV S gene product, as well as initiation and **termination** signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of the vaccinated individual. In some embodiments, the gene construct further comprises an enhancer, **Kozak** sequence (GCCGCCATG SEQ ID NO:13), and at least a fragment of the HCV 5' UTR.  
**DETD** . . . The regulatory elements include a promoter and a polyadenylation signal. In addition, other elements, such as an enhancer and a **Kozak** sequence, may also be included in the gene construct.  
**DETD** . . . Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (**CMV**) such as the **CMV** immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as . . .  
**DETD** . . . but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from **CMV**, RSV and EBV.  
**DETD** In expression vectors of the invention, nucleotide coding sequence encoding the fusion protein is under the regulatory control of the **CMV** immediate early promoter and the SV40 minor polyadenylation signal. Constructs may optionally contain the SV40 origin of replication.  
**DETD** . . . and 2C154XBA-R 5'-TCTCTAGATTACTAGCCATGCGCCAAGGCCCTGG-3' (SEQ ID NO:9) primer. The PCR product was digested with BamHI and XbaI, and ligated into the **pcDNA3** vector (Invitrogen). The final 20 amino acids of the HCV core protein binds tightly to ER membrane. Considering this

digested with BamHI and NaeI and ligated into BamHI site-EcoRI site in the **pcDNA3** vector.

DETD . . . (SEQ ID NO:12). Amplified DNA was digested with BamHI and HindIII, and ligated into the BamHI site-HindIII site of the **pcDNA3** vector that included the HCV core gene. The gene encoding the small S was produced by digestion of middle S. . . (pre S2-S) PCR product by Xho-I followed by Klenow treatment. In the upstream sequence of the pre-S2-S-HCV fusion constructs, a **Kozak** sequence (GCCGCCATG SEQ ID NO:13) was included in the Kz Hind pS2 primer and this was added to the preS2-S-HCV. . .

DETD . . . proteins described above each contain the nucleotide coding region for the fusion protein placed under the transcriptional control of the **CMV** promoter and the RSV enhancer element.

DETD . . . and operably linked to the promoter and polyadenylation signal. Transcription of the cloned inserts is under the control of the **CMV** promoter and the RSV enhancer elements. A polyadenylation signal is provided by the presence of an SV40 poly A signal. . .

L11 ANSWER 3 OF 3 USPATFULL on STN

1998:36597 Bacteriophage-mediated gene transfer systems capable of transfecting eukaryotic cells.

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US 5736388 19980407

**APPLICATION: US 1994-366522 19941230 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

CLM What is claimed is:

1. A bacteriophage lambda particle comprising bacteriophage lambda structural proteins and a nucleic acid molecule comprising a nucleotide sequence exogenous to bacteriophage lambda wherein said bacteriophage structural proteins comprise a modified gpJ tail fiber protein which can bind to the membrane of an animal cell thereby causing said bacteriophage particle to bind said animal cell, and wherein said nucleic acid molecule is taken into said animal cell.

2. The bacteriophage lambda particle according to claim 1 wherein the exogenous nucleotide sequence codes for a compound selected from the group consisting of a polypeptide, an anti-sense RNA, and a ribozyme.

3. A method of producing the bacteriophage lambda particle of claim 1 comprising a) obtaining a head extract comprising said nucleic acid molecule comprising a nucleotide sequence exogenous to bacteriophage lambda in a prohead; b) obtaining a tail extract comprising said modified lambda gpJ bacteriophage tail fiber polypeptide and; c) combining in vitro the head extract and the tail extract to form said bacteriophage lambda particle.

4. The method of claim 3, wherein said exogenous nucleotide sequence codes for a for a compound selected from the group consisting of a polypeptide, an anti-sense RNA, and a ribozyme.

**AI US 1994-366522 19941230 (8)**

DETD . . . antibiotic such as neomycin, hygromycin, phleomycin, histidinol, or methotrexate, as well as one or more restriction sites and a translation **termination** sequence. In addition, if the vector construct is placed into a bacteriophage such as lambda, the vector construct must include. . .

DETD . . . Formation of the initiator complex allows polymerization of gpV, the major tail protein. The addition of gpU and gpZ provide **termination** and maturation functions and generate a mature tail which can bind head structures (Hendrix et al., 1983).

DETD . . . construct may also include a signal which directs polyadenylation, as well as one or more restriction sites and a translation **termination** sequence. In addition, nucleic acid molecules coding for a selectable marker are neither required nor preferred.

DETD . . . used in the vectors of the invention. These include, but are not limited to, the cytomegalovirus major immediate early promoter (**CMV** MIE), the early and late SV40 promoters, the adenovirus major late promoter, thymidine kinase or thymidylate synthase promoters,  $\alpha$  or. . .

DETD Vectors according to the invention may also contain an enhancer sequence, e.g., a **CMV** or SV40 enhancer operably associated with other elements employed to regulate expression. A variety of other elements which control gene. . . from Mason-Pfizer monkey virus (MPMV), a 219 nucleotide sequence that allows rev-independent replication of rev-negative HIV proviral clones, and a **Kozak** sequence. Rev protein functions to allow nuclear export of unspliced and singly spliced HIV RNA molecules. The MPMV element allows. . .

DETD . . . group consisting of influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and **CMV**. Within one

encodes a protein selected from the group consisting of E5, . . .  
 DETD . . . are complementary to influenza virus, HPV, HBV, HCV, EBV, HIV,  
 HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and **CMV** sequences.  
 DETD . . . non structural proteins, a vital junction region, a  
 heterologous sequence, a Sindbis RNA polymerase recognition sequence,  
 and a 3' transcription **termination** polyadenylation signal sequence.  
 The eukaryotic Sindbis cDNA expression vector may include also  
 intervening sequences (introns), which are spliced from the. . .  
 DETD . . . from cDNA is selected from the group consisting of the MoMLV  
 promoter, metallothionein promoter, glucocorticoid promoter, SV40  
 promoter, and the **CMV** promoter. Within further embodiments, the  
 eukaryotic layered vector initiation systems further comprise a  
 polyadenylation sequence.  
 DETD . . . mouse polyoma virus promoter (Py), rous sarcoma virus (RSV), BK  
 virus and JC virus promoters, MMTV promoter, alphavirus junction region,  
**CMV** MIE promoter, adenovirus VA1RNA, rRNA promoter, tRNA methionine  
 promoter and the lac promoter. The second layer comprises a construct  
 which. . .  
 DETD . . . for example, vector systems derived from viruses from the  
 families: Picornaviridae (e.g., poliovirus, rhinovirus,  
 coxsackieviruses), Caliciviridae, Togaviridae (e.g. alphavirus,  
 rubella), **Flaviviridae** (e.g., yellow fever), Coronaviridae (e.g., HCV,  
 TGEV, IBV, MHV, BCV), Rhabdoviridae, Filoviridae, Paramyxoviridae (e.g.,  
 parainfluenza virus, mumps virus, measles virus,. . .  
 DETD . . . polyadenylation signal is provided at a restriction site  
 downstream of the neo<sup>R</sup> insertion site by incorporating bovine  
 growth hormone transcription **termination** sequences from the plasmid  
**pcDNA3** (Invitrogen, San Diego, Calif.) into a downstream site. The  
 nucleic acid molecule comprising the SV40 promoter sequences, the  
 neo<sup>R</sup> gene. . .  
 DETD . . . of bacteriophage transduction of eukaryotic cells can be  
 assayed using a lambda test vector encoding a SV40-neo cassette and/or a  
**CMV-β**-galactosidase construct packaged using wild type head and  
 tail extracts. The bacteriophage may be titered on E. coli and HT-lamB  
 in. . .

=> d his

(FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006)

FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

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      E CHANG G J J/IN
      E CHANG GWONG J J/IN
L1      2 S E4
      E KONISHI E/AU
      E KONISHI E/IN
L2      24958 S CMV OR CMV-IE
L3      3918 S L2 AND KOZAK
L4      3136 S L3 AND TERMINATION
L5      0 S L4 AND (POLY W A)
L6      760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR
L7      33 S L6 AND CMV/CLM
L8      11 S L7 AND AY<1999
L9      498 S L6 AND PCDNA3?
L10     9 S L9 AND AY<1999
L11     3 S L10 NOT L8

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=> e schmaljohn c s/in

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E1      1      SCHMALJOHN ALAN/IN
E2      11     SCHMALJOHN ALAN L/IN
E3      0 --> SCHMALJOHN C S/IN
E4      12     SCHMALJOHN CONNIE S/IN
E5      1      SCHMALL DAVID/IN
E6      2      SCHMALL KARL H/IN
E7      19     SCHMALL KARL HEINZ/IN
E8      1      SCHMALL MARKUS/IN
E9      3      SCHMALL ROBERT A/IN
E10     2      SCHMALLEGGER HELMUT/IN
E11     1      SCHMALLEGGER HERMANN/IN
E12     5      SCHMALLEGGER PETER/IN

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=> s e4

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L12      12 "SCHMALJOHN CONNIE S"/IN

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=> d l12,cbib,ab,clm,1-12

L12 ANSWER 1 OF 12 USPATFULL on STN

2005:171780 Nucleic acid immunization.

**Schmaljohn, Connie S.**, Fort Detrick, MD, UNITED STATES

Fuller, James T., Middleton, WI, UNITED STATES

Powderject Vaccines, Inc., Madison, WI, UNITED STATES (U.S. corporation)

- AB Recombinant nucleic acid molecules are described. The molecules have a sequence or sequences encoding an antigen from *Bacillus anthracis*. Vectors and compositions containing these molecules are also described. Methods for eliciting an immune response using these molecules and compositions are also described.
- CLM What is claimed is:
1. A polynucleotide vaccine composition comprising a nucleic acid sequence that encodes a *Bacillus anthracis* antigen, wherein said nucleic acid sequence is operatively linked to a promoter suitable for expression of the antigen in a mammalian cell.
  2. The composition of claim 1 wherein the nucleic acid sequence is present in a plasmid vector.
  3. The composition of claim 1 wherein the nucleic acid sequence encodes an antigen obtained or derived from the Protective Antigen of *Bacillus anthracis*.
  4. The composition of claim 3 wherein the antigen encoded by the nucleic acid sequence is substantially homologous to the full-length Protective Antigen protein.
  5. A polynucleotide vaccine composition, said composition comprising: a first nucleic acid sequence that encodes a *Bacillus anthracis* antigen; and a second nucleic acid sequence that encodes a leader signal peptide operatively linked to the first nucleic acid sequence, wherein said first and said second nucleic acid sequences are operatively linked to a promoter suitable for expression thereof in a mammalian cell and said leader signal peptide provides for the secretion of the encoded antigen.
  6. The composition of claim 1 further comprising an adjuvant component.
  7. The composition of claim 6 wherein said adjuvant component is present in the composition in the form of a nucleic acid sequence.
  8. The composition of claim 7 wherein said adjuvant component is a CpG sequence.
  9. The composition of claim 7 wherein said adjuvant component is a further nucleic acid sequence that encodes a polypeptide adjuvant.
  10. The composition of claim 6 wherein said adjuvant component is present in the composition in a form other than a nucleic acid sequence.
  11. The composition of claim 10 wherein said adjuvant component is selected from the group consisting of a polypeptide, a lipid, a non-protein hormone, and a vitamin.
  12. The composition of claim 11 wherein the adjuvant component comprises monophosphoryl lipid A.
  13. The composition of claim 11 wherein the adjuvant component comprises a saponin or a derivative thereof.
  14. The composition of claim 13 wherein the adjuvant component comprises Quil-A.
  15. The composition of claim 1 further comprising a pharmaceutically acceptable excipient or vehicle.
  16. The composition of claim 1 wherein said composition is in particulate form.
  17. The composition of claim 16 wherein the nucleic acid sequence is coated onto a core carrier particle.
  18. The composition of claim 17 wherein the core carrier particle has an average diameter of about 0.1 to about 10  $\mu\text{m}$ .
  19. The composition of claim 17 wherein the core carrier particle comprises a metal.
  20. The composition of claim 19 wherein the metal is gold.
  21. The composition of claim 1 further comprising a transfection facilitating agent.
  22. The composition of claim 5 further comprising an adjuvant component.

present in the composition in the form of a nucleic acid sequence.

24. The composition of claim 23 wherein said adjuvant component is a CpG sequence.

25. The composition of claim 23 wherein said adjuvant component is a further nucleic acid sequence that encodes a polypeptide adjuvant.

26. The composition of claim 5 wherein said adjuvant component is present in the composition in a form other than a nucleic acid sequence.

27. The composition of claim 26 wherein said adjuvant component is selected from the group consisting of a polypeptide, a lipid, a non-protein hormone, and a vitamin.

28. The composition of claim 27 wherein the adjuvant component comprises monophosphoryl lipid A.

29. The composition of claim 27 wherein the adjuvant component comprises a saponin or a derivative thereof.

30. The composition of claim 29 wherein the adjuvant component comprises Quil-A.

31. The composition of claim 5 further comprising a pharmaceutically acceptable excipient or vehicle.

32. The composition of claim 5 wherein said composition is in particulate form.

33. The composition of claim 32 wherein the nucleic acid sequence is coated onto a core carrier particle.

34. The composition of claim 33 wherein the core carrier particle has an average diameter of about 0.1 to about 10  $\mu\text{m}$ .

35. The composition of claim 34 wherein the core carrier particle comprises a metal.

36. The composition of claim 35 wherein the metal is gold.

37. The composition of claim 5 further comprising a transfection facilitating agent.

38. A method for eliciting an immune response against *Bacillus anthracis* in a subject, the method comprising administering the vaccine composition of claim 5 to the subject, whereby upon introduction to the subject, the nucleic acid sequence is expressed to provide the *Bacillus anthracis* antigen in an amount sufficient to elicit said immune response.

39. The method of claim 38 wherein the vaccine composition is administered directly into skin or muscle tissue.

40. The method of claim 38 wherein the vaccine composition is administered to the subject in particulate form.

41. The method of claim 38 wherein the nucleic acid sequence is coated onto a core carrier particle and administered to the subject using a particle-mediated delivery technique.

42. The method of claim 38 wherein the vaccine composition further comprises an adjuvant component.

43. The method of claim 38 further comprising the step of administering a second vaccine composition to the subject.

44. The method of claim 43 wherein the second vaccine composition is an anti-*Bacillus anthracis* vaccine containing the peptide form of the Protective Antigen from *Bacillus anthracis*.

45. The method of claim 43 wherein the second vaccine composition is administered to the subject in a boosting step.

46. The method of claim 43 wherein both vaccine compositions are administered to the same site in the subject.

47. The method of claim 43 wherein the vaccine compositions are administered concurrently.

48. The method of claim 43 wherein the vaccine compositions are combined to provide a single composition.

49. A method for using a Bacillus anthracis antigen to induce a protective immune response in a subject, said method comprising: (a) providing an expression cassette containing a nucleic acid sequence encoding the Protective Antigen from Bacillus anthracis operatively linked to control sequences that direct expression of the Protective Antigen when introduced into tissue of the subject; and (b) administering the expression cassette to tissue of the subject such that the Protective Antigen is expressed in an amount sufficient to induce said protective immune response in the subject.

50. The method of claim 49 wherein the expression cassette is present in a plasmid vector.

51. A method for using a Bacillus anthracis antigen to induce an immune response in a subject, said method comprising: (a) providing an expression cassette containing a first nucleic acid sequence encoding the Protective Antigen from Bacillus anthracis and a second nucleic acid sequence that encodes a leader signal peptide, wherein said first and second nucleic acid sequences are operatively linked to each other and to control sequences that direct expression of said sequences when introduced into tissue of the subject and said leader signal peptide provides for the secretion of the encoded Protective Antigen; and (b) administering the expression cassette to tissue of the subject such that the Protective Antigen is expressed in an amount sufficient to induce said immune response in the subject.

52. The method of claim 51 wherein the leader signal peptide is the tissue plasminogen activator (TPA) leader signal peptide.

53. The method of claim 51 wherein the expression cassette is present in a plasmid vector.

54. The method of claim 53 wherein the plasmid vector is administered directly into skin or muscle tissue of the subject.

55. The method of claim 53 wherein the plasmid vector is administered to the subject in particulate form.

56. The method of claim 55 wherein the plasmid vector is coated onto a core carrier particle and administered to the subject using a particle-mediated delivery technique.

L12 ANSWER 2 OF 12 USPATFULL on STN

2004:108128 Nucleic acid immunization.

Schmaljohn, Connie S., Fort Detrick, MD, UNITED STATES

Fuller, James T., Middleton, WI, UNITED STATES

US 2004082530 A1 20040429

APPLICATION: US 2003-411205 A1 20030411 (10)

PRIORITY: US 2002-371416P 20020411 (60)

DOCUMENT TYPE: Utility; APPLICATION.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Recombinant nucleic acid molecules are described. The molecules have a sequence or sequences encoding an antigen from Bacillus anthracis. Vectors and compositions containing these molecules are also described. Methods for eliciting an immune response using these molecules and compositions are also described.

CLM What is claimed is:

1. A polynucleotide vaccine composition comprising a nucleic acid sequence that encodes a Bacillus anthracis antigen, wherein said nucleic acid sequence is operatively linked to a promoter suitable for expression of the antigen in a mammalian cell.

2. The composition of claim 1 wherein the nucleic acid sequence is present in a plasmid vector.

3. The composition of claim 1 wherein the nucleic acid sequence encodes an antigen obtained or derived from the Protective Antigen of Bacillus anthracis.

4. The composition of claim 3 wherein the antigen encoded by the nucleic acid sequence is substantially homologous to the full-length Protective Antigen protein.

5. A polynucleotide vaccine composition, said composition comprising: a first nucleic acid sequence that encodes a Bacillus anthracis antigen; and a second nucleic acid sequence that encodes a leader signal peptide operatively linked to the first nucleic acid sequence, wherein said first and said second nucleic acid sequences are operatively linked to a promoter suitable for expression thereof in a mammalian cell and said leader signal peptide provides for the secretion of the encoded antigen.

7. The composition of claim 6 wherein said adjuvant component is present in the composition in the form of a nucleic acid sequence.
8. The composition of claim 7 wherein said adjuvant component is a CpG sequence.
9. The composition of claim 7 wherein said adjuvant component is a further nucleic acid sequence that encodes a polypeptide adjuvant.
10. The composition of claim 6 wherein said adjuvant component is present in the composition in a form other than a nucleic acid sequence.
11. The composition of claim 10 wherein said adjuvant component is selected from the group consisting of a polypeptide, a lipid, a non-protein hormone, and a vitamin.
12. The composition of claim 11 wherein the adjuvant component comprises monophosphoryl lipid A.
13. The composition of claim 11 wherein the adjuvant component comprises a saponin or a derivative thereof.
14. The composition of claim 13 wherein the adjuvant component comprises Quil-A.
15. The composition of claim 1 further comprising a pharmaceutically acceptable excipient or vehicle.
16. The composition of claim 1 wherein said composition is in particulate form.
17. The composition of claim 16 wherein the nucleic acid sequence is coated onto a core carrier particle.
18. The composition of claim 17 wherein the core carrier particle has an average diameter of about 0. to about 10  $\mu\text{m}$ .
19. The composition of claim 17 wherein the core carrier particle comprises a metal.
20. The composition of claim 19 wherein the metal is gold.
21. The composition of claim 1 further comprising a transfection facilitating agent.
22. The composition of claim 5 further comprising an adjuvant component.
23. The composition of claim 22 wherein said adjuvant component is present in the composition in the form of a nucleic acid sequence.
24. The composition of claim 23 wherein said adjuvant component is a CpG sequence.
25. The composition of claim 23 wherein said adjuvant component is a further nucleic acid sequence that encodes a polypeptide adjuvant.
26. The composition of claim 5 wherein said adjuvant component is present in the composition in a form other than a nucleic acid sequence.
27. The composition of claim 26 wherein said adjuvant component is selected from the group consisting of a polypeptide, a lipid, a non-protein hormone, and a vitamin.
28. The composition of claim 27 wherein the adjuvant component comprises monophosphoryl lipid A.
29. The composition of claim 27 wherein the adjuvant component comprises a saponin or a derivative thereof.
30. The composition of claim 29 wherein the adjuvant component comprises Quil-A.
31. The composition of claim 5 further comprising a pharmaceutically acceptable excipient or vehicle.
32. The composition of claim 5 wherein said composition is in particulate form.
33. The composition of claim 32 wherein the nucleic acid sequence is coated onto a core carrier particle.

average diameter of about 0.1 to about 10  $\mu\text{m}$ .

35. The composition of claim 34 wherein the core carrier particle comprises a metal.

36. The composition of claim 35 wherein the metal is gold.

37. The composition of claim 5 further comprising a transfection facilitating agent.

38. A method for eliciting an immune response against *Bacillus anthracis* in a subject, the method comprising administering the vaccine composition of claim 5 to the subject, whereby upon introduction to the subject, the nucleic acid sequence is expressed to provide the *Bacillus anthracis* antigen in an amount sufficient to elicit said immune response.

39. The method of claim 38 wherein the vaccine composition is administered directly into skin or muscle tissue.

40. The method of claim 38 wherein the vaccine composition is administered to the subject in particulate form.

41. The method of claim 38 wherein the nucleic acid sequence is coated onto a core carrier particle and administered to the subject using a particle-mediated delivery technique.

42. The method of claim 38 wherein the vaccine composition further comprises an adjuvant component.

43. The method of claim 38 further comprising the step of administering a second vaccine composition to the subject.

44. The method of claim 43 wherein the second vaccine composition is an anti-*Bacillus anthracis* vaccine containing the peptide form of the Protective Antigen from *Bacillus anthracis*.

45. The method of claim 43 wherein the second vaccine composition is administered to the subject in a boosting step.

46. The method of claim 43 wherein both vaccine compositions are administered to the same site in the subject.

47. The method of claim 43 wherein the vaccine compositions are administered concurrently.

48. The method of claim 43 wherein the vaccine compositions are combined to provide a single composition.

49. A method for using a *Bacillus anthracis* antigen to induce a protective immune response in a subject, said method comprising: (a) providing an expression cassette containing a nucleic acid sequence encoding the Protective Antigen from *Bacillus anthracis* operatively linked to control sequences that direct expression of the Protective Antigen when introduced into tissue of the subject; and (b) administering the expression cassette to tissue of the subject such that the Protective Antigen is expressed in an amount sufficient to induce said protective immune response in the subject.

50. The method of claim 49 wherein the expression cassette is present in a plasmid vector.

51. A method for using a *Bacillus anthracis* antigen to induce an immune response in a subject, said method comprising: (a) providing an expression cassette containing a first nucleic acid sequence encoding the Protective Antigen from *Bacillus anthracis* and a second nucleic acid sequence that encodes a leader signal peptide, wherein said first and second nucleic acid sequences are operatively linked to each other and to control sequences that direct expression of said sequences when introduced into tissue of the subject and said leader signal peptide provides for the secretion of the encoded Protective Antigen; and (b) administering the expression cassette to tissue of the subject such that the Protective Antigen is expressed in an amount sufficient to induce said immune response in the subject.

52. The method of claim 51 wherein the leader signal peptide is the tissue plasminogen activator (TPA) leader signal peptide.

53. The method of claim 51 wherein the expression cassette is present in a plasmid vector.

54. The method of claim 53 wherein the plasmid vector is administered



55. The method of claim 53 wherein the plasmid vector is administered to the subject in particulate form.

56. The method of claim 55 wherein the plasmid vector is coated onto a core carrier particle and administered to the subject using a particle-mediated delivery technique.

L12 ANSWER 3 OF 12 USPATFULL on STN

2004:69989 DNA vaccines against hantavirus infections.

Hooper, Jay W., New Market, MD, UNITED STATES

Schmaljohn, Connie S., Frederick, MD, UNITED STATES

Custer, Max, Durham, NC, UNITED STATES

US 2004053216 A1 20040318

APPLICATION: US 2003-394388 A1 20030321 (10)

PRIORITY: US 2002-367128P 20020322 (60)

US 2002-398985P 20020726 (60)

DOCUMENT TYPE: Utility; APPLICATION.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB In this application is described a protective DNA vaccines against infection with HFRS- and HPS-associated hantaviruses. The vaccines were constructed by subcloning cDNA representing the medium (M) (encoding the G1 and G2 glycoproteins) into the DNA expression vector pWRG7077. Animals vaccinated with the M construct developed a neutralizing antibody response. Passive transfer experiments show that serum from vaccinated animals, when injected on days 4 or 5 after challenge, protected animals from lethal disease.

CLM What is claimed is:

1. A nucleic acid set forth in SEQ ID NO: 6.
2. A DNA fragment which encodes a Hantaan hantavirus M gene segment and the sequence according to claim 1.
3. A DNA fragment which encodes a Seoul hantavirus M gene segment and the sequence according- to claim 1.
4. A DNA fragment which encodes an Andes hantavirus M gene segment and the sequence according to claim 1.
5. A recombinant DNA construct comprising: (i) a vector, (ii) at least one hantavirus M gene nucleic acid fragment, and (iii) the nucleic acid fragment of claim 1.
6. The recombinant DNA construct of claim 5 wherein said construct is pWRG/SEO-M set forth in SEQ ID NO:1.
7. The recombinant DNA construct of claim 5 wherein said construct is pWRG/HTN-M(x) set forth in SEQ ID NO:7.
8. The recombinant DNA construct of claim 5 wherein said construct is pWRG/AND-M(x) set forth in SEQ ID NO:8.
9. The recombinant DNA construct of claim 5 wherein said construct comprises two said hantavirus M gene nucleic acid fragments.
10. The recombinant DNA construct of claim 9 wherein said construct is pWRG/HA-M set forth in SEQ NO:9.
11. A composition of matter comprising inert particles; and a nucleic acid coated onto the inert particles producing nucleic acid coated particles, said nucleic acid comprising a promoter operative in the cells of a mammal and a hantavirus polynucleotide M segment encoding G1 and G2 from one or more hantavirus.
12. The composition of claim 11 wherein said hantavirus is chosen from the group consisting of Seoul virus, Dobrava virus, Puumala virus, Hantaan virus, Sin Nombre virus, Black Creek Canal virus, Bayou virus, New York virus, Andes virus, and Laguna Negra virus.
13. The composition of claim 11 wherein said nucleic acid further comprises a sequence set forth in SEQ ID NO:6.
14. The composition of claim 13 wherein said hantavirus is SEOV.
15. The composition of claim 14 wherein said nucleic acid is pWRG/SEO-M set forth in SEQ ID NO:1.
16. The composition of claim 13 wherein said hantavirus is HTNV.
17. The composition of claim 16 wherein said nucleic acid is pWRG/HTN-M(x) set forth in SEQ ID NO:7.

18. The composition of claim 13 wherein said hantavirus is ANDV.
19. The composition of claim 18 wherein said nucleic acid is pWRG/AND-M set forth in SEQ ID NO:8.
20. The composition of claim 11 wherein the nucleic acid comprises M segment from two hantavirus.
21. The composition of claim 20 wherein said two hantavirus are HTNV and ANDV.
22. The composition of claim 21 wherein said nucleic acid is pWRG/HA-M set forth in SEQ ID NO:9.
23. A method for inducing a protective immune response against hantavirus infection in a mammal, comprising accelerating the composition according to claim 11 into epidermal cells of the mammal in vivo such that said nucleic acid is expressed.
24. A method for inducing a protective immune response against SEOV, HTNV, DOBV hantavirus infection in a mammal, comprising accelerating the composition according to claim 15 into epidermal cells of the mammal in vivo such that said nucleic acid is expressed.
25. A method for inducing a protective immune response against HTNV, SEOV, and DOBV hantavirus infection in a mammal, comprising accelerating the composition according to claim 17 into epidermal cells of the mammal in vivo such that said nucleic acid is expressed.
26. A method for inducing a protective immune response against ANDV, SNV, and BCCV hantavirus infection in a mammal, comprising accelerating the composition according to claim 19 into epidermal cells of the mammal in vivo such that said nucleic acid is expressed.
27. A method for inducing a protective immune response against HTNV, SEOV, DOBV, ANDV, SNV, and BCCV hantavirus infection in a mammal, comprising accelerating the composition according to claim 22 into epidermal cells of the mammal in vivo such that said nucleic acid is expressed.
28. A vaccine against infection with SEOV, DOBV, and HTNV hantavirus, said vaccine comprising the composition of claim 15.
29. A vaccine against infection with SEOV, DOBV, and HTNV hantavirus, said vaccine comprising the composition of claim 17.
30. A vaccine against infection with ANDV, SNV, and BCCV hantavirus, said vaccine comprising the composition of claim 19.
31. A vaccine against infection with SEOV, HTNV, DOBV, ANDV, SNV, and BCCV, said vaccine comprising the composition of claim 22.
32. A multivalent vaccine for protection against infection with more than one HFRS hantavirus comprising a composition of matter comprising an inert particle having a nucleic acid coated onto said particle, said nucleic acid comprising two or more hantavirus M segments from different hantaviruses, each M segment encoding G1 and G2 from its respective hantavirus, operatively linked to a promoter active in cells of a mammal.
33. The multivalent vaccine of claim 32 wherein said hantaviruses are chosen from the group SEOV, PUUV, HTNV and DOBV.
34. A multivalent vaccine for protection against infection with more than one HFRS hantavirus comprising a composition of matter comprising two or more inert particles, each said particle having a nucleic acid coated onto, said nucleic acid comprising a hantavirus M segment from HFRS-associated hantavirus encoding G1 and G2 and operatively linked to a promoter active in cells of a mammal, wherein said M segment is chosen from different hantaviruses.
35. The multivalent vaccine according to claim 34 wherein said hantaviruses are chosen from the group consisting of HTNV, DOBV, PUUV and SEOV.
36. A multivalent vaccine for protection against infection with more than one HPS hantavirus comprising a composition of matter comprising an inert particle having a nucleic acid coated onto said particle, said nucleic acid comprising two or more hantavirus M segments from different HPS-associated hantaviruses, each M segment encoding G1 and G2 from its respective hantavirus, operatively linked to a promoter active in cells of a mammal.

37. The multivalent vaccine according to claim 36 wherein said hantavirus is chosen from the group consisting of ANDV, SNV, and BCCV.

38. A multivalent vaccine for protection against infection with more than one HPS hantavirus comprising a composition of matter comprising two or more inert particles, each said particle having a nucleic acid coated onto, said nucleic acid comprising a hantavirus M segment from HPS-associated hantavirus encoding G1 and G2 and operatively linked to a promoter active in cells of a mammal, wherein said M segment is chosen from different hantaviruses.

39. The multivalent vaccine according to claim 38 wherein said hantavirus is chosen from the group consisting of ANDV, SNV, and BCCV.

40. A multivalent vaccine for protection against infection with more than one HFRS and HPS hantavirus comprising a composition of matter comprising two or more inert particles, each said particle having a nucleic acid coated onto, said nucleic acid comprising a hantavirus M segment encoding G1 and G2 and operatively linked to a promoter active in cells of a mammal, wherein said M segment is chosen from different hantaviruses including at least one HPS-associated hantavirus and at least one HFRS-associated hantavirus.

41. The multivalent vaccine of claim 40 wherein said hantaviruses are chosen from the group consisting of ANDV, HTNV, and SEOV.

42. A multivalent vaccine for protection against infection with more than one HFRS and HPS hantavirus comprising a composition of matter comprising an inert particle having two or more nucleic acids coated onto said particle, each said nucleic acids comprising a hantavirus M segment encoding G1 and G2 and operatively linked to a promoter active in cells of a mammal, wherein said M segment is chosen from different hantaviruses including at least one HPS-associated hantavirus and at least one HFRS-associated hantavirus.

43. The multivalent vaccine of claim 42 wherein said hantaviruses are chosen from the group consisting of ANDV, HTNV, and SEOV.

44. A composition comprising polyclonal antibodies from a population of vaccinees vaccinated with a DNA vaccine comprised of a plasmid expressing a hantavirus M gene segment.

45. The composition of claim 44 wherein said hantavirus is chosen from the group consisting of Seoul virus, Dobrava virus, Puumala virus, Hantaan virus, Sin Nombre virus, Black Creek Canal virus, Bayou virus, New York virus, Andes virus, and Laguna Negra virus.

46. The composition of claim 44 wherein said vaccine comprises pWRG/SEO-M.

47. The composition of claim 44 wherein said vaccine comprises pWRG/HTN-M(x).

48. The composition of claim 44 wherein said vaccine comprises pWRG/AND-M.

49. The composition of claim 44 wherein said vaccine comprises pWRG/HA-M.

50. The composition of claim 44 wherein said composition inhibits hantavirus infection in a subject in vivo.

51. The composition of claim 50 wherein said subject is mammalian.

51. The composition of claim 44 wherein said composition ameliorates symptoms of hantavirus infection when said composition is administered to a subject after infection with hantavirus.

52. The composition of claim 51 wherein said subject is mammalian.

53. The composition of claim 44 wherein said polyclonal antibodies neutralize hantavirus in vitro.

54. A therapeutic composition for ameliorating symptoms of hantavirus infection comprising the composition of claim 44, and a pharmaceutically acceptable excipient.

55. A passive vaccine against hantavirus infection comprising the composition of claim 44.

56. An anti-hantavirus composition, comprising polyclonal antibodies from a vaccinee vaccinated with a DNA vaccine comprising the M gene

hantavirus infection, and a pharmaceutically acceptable carrier.

57. A method of treating hantavirus infection comprising administering to a patient in need of said treatment an effective amount of a composition according to claim 54.

58. A method for detecting hantavirus infection comprising contacting a sample from a subject suspected of having hantavirus infection with a antibody according to claim 44 and detecting the presence or absence by detecting the presence or absence of a complex formed between the hantavirus antigens and antibodies specific therefor.

59. A method for the diagnosis of hantavirus infection comprising the steps of: (i) contacting a sample from an individual suspected of having hantavirus infection with a composition according to claim 44; and (ii) detecting the presence or absence of hantavirus infection by detecting the presence or absence of a complex formed between hantavirus antigens and antibodies specific therefor.

60. A hantavirus infection diagnostic kit comprising the composition of claim 44 and ancillary reagents suitable for use in detecting the presence or absence of hantavirus antigens in a sample.

L12 ANSWER 4 OF 12 USPTAFULL on STN

2003:30279 Prophylactic and therapeutic monoclonal antibodies.

Hooper, Jay W., New Market, MD, UNITED STATES

Schmaljohn, Alan L., Frederick, MD, UNITED STATES

**Schmaljohn, Connie S.**, Frederick, MD, UNITED STATES

US 2003022226 A1 20030130

APPLICATION: US 2002-202532 A1 20020916 (10)

PRIORITY: US 2000-182066P 20000211 (60)

DOCUMENT TYPE: Utility; APPLICATION.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB In this application are described vaccinia monoclonal antibodies. Also provided are mixtures of antibodies of the present invention, as well as methods of using individual antibodies or mixtures thereof for the detection, prevention, and/or therapeutical treatment of vaccinia virus infections in vitro and in vivo.

CLM What is claimed is:

1. A composition comprising one or more monoclonal antibody directed against a vaccinia virus antigen.

2. The composition of claim 1 wherein said vaccinia virus antigen is L1R.

3. The composition of claim 1 wherein said vaccinia virus antigen is A33R.

4. The composition of claim 2 wherein said composition further comprises one or more monoclonal antibody directed against vaccinia A33R.

5. The composition of claim 4 wherein said composition further comprises one or more monoclonal antibody directed against an antigen chosen from the group consisting essentially of: vaccinia H3L, D8L, B5R, A27L and A17L.

6. The composition of claim 4 wherein said composition inhibits vaccinia virus infection in a subject in vivo.

7. The composition of claim 6 wherein said subject is avian or mammalian.

8. The composition of claim 4 wherein said composition ameliorates symptoms of vaccinia virus infection when said composition is administered to a subject after infection with vaccinia virus.

9. The composition of claim 8 wherein said subject is avian or mammalian.

10. The composition of claim 2 wherein said monoclonal antibody immunoprecipitates L1R in vitro.

11. The composition of claim 3 wherein said monoclonal antibody immunoprecipitates A33R in vitro.

12. A therapeutic composition for ameliorating symptoms of vaccinia virus infection comprising the composition of claim 4, and a pharmaceutically acceptable excipient.

13. A passive vaccine against vaccinia virus infection comprising the composition of claim 4.

14. An anti-vaccinia composition, comprising one or more monoclonal antibodies, wherein at least two of said monoclonal antibodies are directed against L1R and A33R, in an amount effective for inhibiting vaccinia virus infection, and a pharmaceutically acceptable carrier.

15. A method of treating vaccinia virus infection comprising administering to a patient in need of said treatment an effective amount of a composition according to claim 4.

16. The composition according to claim 1 wherein said vaccinia virus antigen is chosen from the vaccinia strain Connaught, IHD-J, Brighton, WR, Lister, Copenhagen, Ankara, Dairen I, L-IPV, LC16M8, LC16MO, LIVP, Tian Tan, WR 65-16, Wyeth.

17. A poxvirus monoclonal antibody composition comprising monoclonal antibodies against a homolog of a vaccinia antigen chosen from the group consisting of L1R and A33R, said poxvirus chosen from the group consisting of: orthopoxvirus, parapoxvirus, avipoxvirus, capripoxvirus, leporipoxvirus, suipoxvirus, molluscipoxvirus, and yatapoxvirus.

L12 ANSWER 5 OF 12 USPATFULL on STN

2002:343951 Antibodies expressed in insect cells.

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Schmaljohn, Connie S., Frederick, MD, UNITED STATES

US 2002197677 A1 20021226

APPLICATION: US 2002-94546 A1 20020308 (10)

PRIORITY: US 2001-274164P 20010308 (60)

DOCUMENT TYPE: Utility; APPLICATION.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Three vectors are described which allow the conversion of Fab fragments into or single chain Fv fragments into full-length antibody molecules. Methods for using the vectors and the resulting antibodies are also described.

CLM What is claimed is:

1. A DNA construct for expressing in insect cells a complete antibody light chain derived from the corresponding Fab fragment, comprising in 5' to 3' order, a promoter that promotes transcription in insect cells, a signal sequence which directs secretion of a protein from insect cells, a DNA sequence which encodes the variable and constant domain of the antibody light chain derived from a Fab fragment and a transcription terminator functional in insect cells.

2. The DNA construct of claim 1 wherein said signal sequence is a human signal sequence.

3. The DNA construct of claim 2 wherein said human signal sequence is derived from an antibody.

4. The DNA construct of claim 3 wherein the promoter is the baculovirus ieI promoter.

5. The DNA construct of claim 4 wherein the construct further provides restriction enzyme sites for inserting the DNA sequence encoding the variable and constant domain from the desired Fab fragment.

6. The DNA construct of claim 5 wherein said construct further provides a selectable marker.

7. The DNA construct of claim 5 wherein said construct is pIEI-Light.

8. A DNA construct for expressing in insect cells a complete antibody heavy chain derived from the corresponding Fab fragment, comprising in 5' to 3' order, a promoter that promotes transcription in insect cells, a signal sequence which directs secretion of a protein from insect cells, a DNA sequence which encodes the variable domain of the antibody heavy chain derived from a Fab fragment, a complete IgG1 constant region and a transcription terminator functional in insect cells.

9. The DNA construct of claim 8 wherein said signal sequence is a human signal sequence.

10. The DNA construct of claim 9 wherein said human signal sequence is derived from an antibody.

11. The DNA construct of claim 10 wherein the promoter is the baculovirus ieI promoter.

12. The DNA construct of claim 11 wherein the construct further provides restriction enzyme sites for inserting the DNA sequence encoding the variable and constant domain from the desired Fab fragment.

provides a selectable marker.

14. The DNA construct of claim 13 wherein said construct is pIEI-Heavy.

15. A method for obtaining a conformationally active IgG1 antibody from a corresponding Fab fragment comprising: transforming insect cells with (i) a DNA construct for expressing in insect cells a complete antibody light chain derived from the corresponding Fab fragment, comprising in 5' to 3' order, a promoter that promotes transcription in insect cells, a signal sequence which directs secretion of a protein from insect cells, a DNA sequence which encodes the variable and constant domain of the antibody light chain derived from a Fab fragment and a transcription terminator functional in insect cells, and (ii) a DNA construct for expressing in insect cells a complete antibody heavy chain derived from the corresponding Fab fragment, comprising in 5' to 3' order, a promoter that promotes transcription in insect cells, a signal sequence which directs secretion of a protein from insect cells, a DNA sequence which encodes the variable domain of the antibody heavy chain derived from a Fab fragment, a complete IgG1 constant region and a transcription terminator functional in insect cells, selecting transformed insect cells that have acquired the DNA constructs, culturing the transformed insect cells under conditions such that the IgG1 antibody is produced and secreted, and isolating the IgG1 antibody.

16. The method of claim 15 wherein the DNA construct in (i) is pIEI-Light and the construct in (ii) is pIEI-Heavy.

17. The method of claim 15 wherein the insect cells are TN cells.

18. An insect cell transformed with the DNA construct pIEI-Light.

19. An insect cell transformed with the DNA construct pIEI-Heavy.

20. An insect cell transformed with the DNA constructs pIEI-Light and pIEI-Heavy.

21. An insect cell expressing a desired antibody produced by transforming said insect cell with the complete the DNA constructs pIEI-Light and pIEI-Heavy.

22. An IgG1 antibody derived from the expression in an insect cell of (i) a DNA construct for expressing in insect cells a complete antibody light chain derived from the corresponding Fab fragment, comprising in 5' to 3' order, a promoter that promotes transcription in insect cells, a signal sequence which directs secretion of a protein from insect cells, a DNA sequence which encodes the variable and constant domain of the antibody light chain derived from a Fab fragment and a transcription terminator functional in insect cells, and (ii) a DNA construct for expressing in insect cells a complete antibody heavy chain derived from the corresponding Fab fragment, comprising in 5' to 3' order, a promoter that promotes transcription in insect cells, a signal sequence which directs secretion of a protein from insect cells, a DNA sequence which encodes the variable domain of the antibody heavy chain derived from a Fab fragment, a complete IgG1 constant region and a transcription terminator functional in insect cells.

23. The antibody of claim 22 wherein said DNA construct in (i) is pIEI-Light and the DNA construct in (ii) is pIEI-Heavy.

24. A therapeutic composition comprising the antibody of claim 23, and a pharmaceutically acceptable excipient.

25. A passive vaccine against an infectious agent recognized by the antibody of claim 23, said vaccine comprising the antibody of claim 23.

26. A method for detecting an infectious agent in a sample, said method comprising: (i) incubating the sample with an effective amount of at least one antibody according to claim 22, said antibody able to recognize said agent, under conditions which allow the formation of an antibody-agent complex; and (ii) detecting the antibody-agent complex wherein the presence or absence of the complex indicates the presence or absence of the agent in the sample.

27. A method for detecting an infectious agent according to claim 26, wherein said sample is a biological sample.

28. A method of treating an infection caused by an agent recognized by an antibody according to claim 22, comprising administering to a patient in need of said treatment an effective amount of a composition comprising one or more antibodies.

29. A kit for detecting an infectious agent in a biological sample, said

antibody according to claim 22 which recognizes said agent, and (2) instructions for using said antibody for the purpose of binding to said agent to form an immunological complex and detecting the formation of the immunological complex such that presence or absence of immunological complex correlates with presence or absence of said agent in said sample.

30. A DNA construct for expressing in insect cells a complete antibody light chain derived from the corresponding single chain Fv, comprising in 5' to 3' order, a transcription terminator functional in insect cells, a human constant light chain region, a DNA sequence which encodes a variable domain of the antibody light chain derived from a single chain Fv fragment, a signal sequence which directs secretion of a protein from insect cells, and a promoter that promotes transcription in insect cells.

31. The DNA construct of claim 30 wherein said human constant light chain region is a lambda light chain.

32. The DNA construct of claim 30 wherein said human constant light chain region is a kappa light chain.

33. The DNA construct of claim 31 wherein said signal sequence is a human signal sequence.

34. The DNA construct of claim 33 wherein said promoter is a p10 promoter.

35. The DNA construct of claim 34 further providing enzyme restriction sites convenient for inserting the desired human light chain variable region.

36. The DNA construct of claim 35 wherein said construct is pAc-V-Light.

37. A method for obtaining a conformationally active IgG1 antibody from a corresponding single chain Fv antibody fragment comprising: producing a complete chimeric light chain gene by inserting the light chain variable region from scFv 3' to a constant domain for lambda light chain, transforming insect cells with: (i) a DNA construct comprising the complete chimeric light chain gene, a human constant light chain region, a signal sequence which directs secretion of a protein from insect cells, a promoter that promotes transcription in insect cells, and a transcription terminator functional in insect cells, and (ii) a DNA construct for expressing in insect cells a complete antibody heavy chain derived from the corresponding Fab fragment, comprising in 5' to 3' order, a promoter that promotes transcription in insect cells, a signal sequence which directs secretion of a protein from insect cells, a DNA sequence which encodes the variable domain of the antibody heavy chain derived from a Fab fragment, a complete IgG1 constant region and a transcription terminator functional in insect cells, selecting transformed insect cells that have acquired the DNA constructs, culturing the transformed insect cells under conditions such that the IgG1 antibody is produced and secreted, and isolating the IgG1 antibody.

38. The method of claim 37 wherein said producing of complete light chain is by insertion of the variable region from scFv into a DNA construct pAc-V-Light.

39. The method of claim 38 wherein the insect cells are TN cells.

40. An insect cell transformed with the DNA construct containing the complete light chain produced from scFv and pIE1-Heavy.

41. An insect cell expressing a desired antibody produced by transforming said insect cell with DNA construct containing the complete light chain produced from scFv and pIE1-Heavy.

42. An IgG1 antibody derived from the expression in an insect cell of (i) a DNA construct for expressing in insect cells a complete antibody light chain derived from the corresponding single chain Fv, comprising in 5' to 3' order, a human constant light chain region, a DNA sequence which encodes a partial variable domain of the antibody light chain derived from a single chain Fv fragment, a signal sequence which directs secretion of a protein from insect cells, a promoter that promotes transcription in insect cells, and a transcription terminator functional in insect cells, and (ii) a DNA construct for expressing in insect cells a complete antibody heavy chain derived from the corresponding Fab fragment, comprising in 5' to 3' order, a promoter that promotes transcription in insect cells, a signal sequence which directs secretion of a protein from insect cells, a DNA sequence which encodes the variable domain of the antibody heavy chain derived from an Fab

functional in insect cells.

43. The antibody of claim 42 wherein said DNA construct in (i) is pAc-V-Light and the DNA construct in (ii) is pIE1-Heavy.

44. A therapeutic composition comprising the antibody of claim 43, and a pharmaceutically acceptable excipient.

45. A passive vaccine against an infectious agent recognized by the antibody of claim 43, said vaccine comprising the antibody of claim 43.

46. A method for detecting an infectious agent in a sample, said method comprising: (i) incubating the sample with an effective amount of at least one antibody according to claim 43, said antibody able to recognize said agent, under conditions which allow the formation of an antibody-agent complex; and (ii) detecting the antibody-agent complex wherein the presence or absence of the complex indicates the presence or absence of the agent in the sample.

47. A method for detecting an infectious agent according to claim 46, wherein said sample is a biological sample.

48. A method of treating an infection caused by an agent recognized by an antibody according to claim 43, comprising administering to a patient in need of said treatment an effective amount of a composition comprising one or more antibodies.

49. A kit for detecting an infectious agent in a biological sample, said kit comprising: (1) a container holding at least one monoclonal antibody according to claim 43 which recognizes said agent, and (2) instructions for using said antibody for the purpose of binding to said agent to form an immunological complex and detecting the formation of the immunological complex such that presence or absence of immunological complex correlates with presence or absence of said agent in said sample.

50. A kit for obtaining a conformationally active IgG1 antibody from a corresponding single chain Fv antibody fragment comprising: (i) a DNA construct for expressing in insect cells a complete antibody light chain derived from the corresponding single chain Fv, comprising in 5' to 3' order, a transcription terminator functional in insect cells, a human constant light chain region, a DNA sequence which encodes a variable domain of the antibody light chain derived from a single chain Fv fragment, a signal sequence which directs secretion of a protein from insect cells, a promoter that promotes transcription in insect cells, (ii) a DNA construct for expressing in insect cells a complete antibody heavy chain derived from the corresponding Fab fragment, comprising in 5' to 3' order, a promoter that promotes transcription in insect cells, a signal sequence which directs secretion of a protein from insect cells, a DNA sequence which encodes the variable domain of the antibody heavy chain derived from an Fab fragment, a complete IgG1 constant region and a transcription terminator functional in insect cells.

51. The kit of claim 50 further comprising pAc-V-Light for producing the complete light chain.

52. The kit of claim 51 wherein said DNA construct in (ii) is pIE1-Heavy.

53. A kit for obtaining a conformationally active IgG1 antibody from a corresponding Fab fragment comprising: (i) a DNA construct for expressing in insect cells a complete antibody light chain derived from the corresponding Fab fragment, comprising in 5' to 3' order, a promoter that promotes transcription in insect cells, a signal sequence which directs secretion of a protein from insect cells, a DNA sequence which encodes the variable and constant domain of the antibody light chain derived from a Fab fragment and a transcription terminator functional in insect cells, and (ii) a DNA construct for expressing in insect cells a complete antibody heavy chain derived from the corresponding Fab fragment, comprising in 5' to 3' order, a promoter that promotes transcription in insect cells, a signal sequence which directs secretion of a protein from insect cells, a DNA sequence which encodes the variable domain of the antibody heavy chain derived from an Fab fragment, a complete IgG1 constant region and a transcription terminator functional in insect cells.

54. The kit of claim 53 wherein said DNA construct in (i) is pIE1-Light and said DNA construct in (ii) is pIE1-Heavy.



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**Schmaljohn, Connie S.**, Frederick, MD, UNITED STATES  
US 2002176871 A1 20021128  
APPLICATION: US 2001-800632 A1 20010307 (9)  
PRIORITY: US 2000-187608P 20000307 (60)  
DOCUMENT TYPE: Utility; APPLICATION.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB In this application is described a poxvirus naked DNA vaccine which protects animals against poxvirus challenge comprising IMV and EEV nucleic acids from poxvirus. Methods of use of the vaccine and its advantages are described.

CLM What is claimed is:

1. A DNA vaccine against poxviruses comprising at least one nucleic acids encoding an intracellular mature virion antigen and at least one nucleic acid encoding an extracellular enveloped virion antigen of a poxvirus.
2. The DNA vaccine of claim 1 wherein said poxvirus is chosen from the group consisting of: variola virus, monkeypox virus, cowpox virus, orf virus, paravaccinia virus, Tanapoxvirus, Yabapoxvirus and Molluscum contagiosum
3. The vaccine of claim 1 wherein said poxvirus is vaccinia.
4. The vaccine of claim 3 wherein said intracellular mature virion antigen is chosen from the group consisting of: L1R and A27L or a homolog thereof.
5. The vaccine of claim 3 wherein said extracellular mature virion antigen is chosen from the group consisting of: A33R and B5R or a homolog thereof.
6. A method for inducing in a subject an immune response against poxvirus infection comprising administering to said subject an immunologically effective amount of at least one nucleic acid encoding an intracellular mature virion antigen and at least one nucleic acid encoding an extracellular mature virion antigen of said poxvirus in an acceptable diluent.
7. A composition of matter comprising a carrier particle; and a DNA sequence coated onto the carrier particle, the DNA sequence comprising a promoter operative in the cells of a mammal and a protein coding region coding for a poxvirus antigen chosen from the group consisting of: IMV or EEV antigen.
8. The composition of claim 7 wherein said IMV poxvirus antigen is selected from the group consisting of L1R and A27L.
9. The composition of claim 7 wherein said EEV poxvirus antigen is selected from the group consisting of A33R and B5R.
10. A vaccine comprising a composition of matter according to claim 8 and a composition of matter according to claim 9.
11. A method for inducing a protective immune response to a poxvirus in a mammal, comprising (i) preparing a nucleic acid encoding an antigen of poxvirus operatively linked to a promoter operative in cells of a mammal; (ii) coating the nucleic acid in (i) onto carrier particles; (iii) accelerating the coated carrier particles into epidermal cells of the mammal in vivo; and (iv) detecting a protective immune response in said mammal upon exposure to a poxvirus.
12. The method according to claim 11 wherein the carrier particles are gold.
13. The method according to claim 11 wherein the antigen is chosen from the group consisting of IMV antigen and EEV antigen
14. The method according to claim 11 wherein said poxvirus is VACV.
15. A multivalent vaccine for protection against infection with more than one poxvirus comprising a composition of matter comprising a carrier particle having one or more DNA sequence coated onto the carrier particle, the DNA sequence comprising a promoter operative in the cells of a mammal and a nucleic acid coding for an antigen, said antigen selected from the group consisting of an IMV antigen and an EEV antigen, of a first poxvirus said poxvirus selected from the group consisting of Orthopoxvirus, Parapoxvirus, Caripoxvirus, Suipoxvirus, Leporipoxvirus, Avipoxvirus, Yatapoxvirus, Molluscipoxvirus, macropod poxvirus, and crocodilian poxvirus.
16. The multivalent vaccine of claim 15, further comprising a

sequence coated onto the carrier particle, the DNA sequence comprising a promoter operative in the cells of a mammal a nucleic acid coding for an antigen, said antigen selected from the group consisting of IMV antigen and EEV antigen, of a second poxvirus different from said first poxvirus, said second poxvirus selected from the group consisting of Orthopoxvirus, Parapoxvirus, Caripoxvirus, Suipoxvirus, Leporipoxvirus, Avipoxvirus, Yatapoxvirus, Molluscipoxvirus, macropod poxvirus, and crocodilian poxvirus, wherein the nucleic acid coding for an IMV antigen is not on the same carrier particle as the nucleic acid coding for an EEV antigen.

L12 ANSWER 7 OF 12 USPTAFULL on STN

2002:213445 DNA Vaccines against hantavirus infections.

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Hooper, J. W., New Market, MD, UNITED STATES

US 2002114818 A1 20020822

APPLICATION: US 2000-491974 A1 20000127 (9)

PRIORITY: US 1999-117680P 19990129 (60)

DOCUMENT TYPE: Utility; APPLICATION.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Seoul virus (SEOV) is one of four known hantaviruses causing hemorrhagic fever with renal syndrome (HFRS). Candidate naked DNA vaccines for HFRS were constructed by subcloning cDNA representing the medium (M) (encoding the G1 and G2 glycoproteins) or small (S) (encoding the nucleocapsid protein) genome segment of SEOV into the DNA expression vector pWRG7077. We vaccinated BALB/c mice with three doses of the M or S DNA vaccine at 4-week intervals by either gene gun inoculation of the epidermis, or needle inoculation into the gastrocnemius muscle. Both routes of vaccination resulted in antibody responses as measured by ELISA; however, gene gun inoculation elicited a higher frequency of seroconversion, and higher levels of antibodies in individual mice. We vaccinated Syrian hamsters with the M or S construct using the gene gun and found hantavirus-specific antibodies in 5/5 and 4/5 hamsters, respectively. Animals vaccinated with the M construct developed a neutralizing antibody response which was greatly enhanced in the presence of guinea pig complement. Immunized hamsters were challenged with SEOV and, after 28 days, were monitored for evidence of infection. Hamsters vaccinated with M were protected from infection, but hamsters vaccinated with S were not protected.

CLM What is claimed is:

1. A composition of matter comprising a carrier particle; and a DNA sequence coated onto the carrier particle, the DNA sequence comprising a promoter operative in the cells of a mammal and a protein coding region coding for a determinant of a hantavirus protein.

2. The composition of claim 1 wherein said protein coding region encodes a protein selected from the group consisting of M gene segment proteins and S gene segment proteins.

3. The composition of claim 1 wherein said hantavirus is chosen from the group consisting of Seoul virus, Dobrava virus, Puumala virus, Hantaan virus, Sin Nombre virus, Black Creek Canal virus, Bayou virus, New York virus, Andes virus, and Laguna Negra virus.

4. The composition of claim 3 wherein said hantavirus is Seoul virus.

5. The composition of claim 4 wherein the protein coding region comprises SEQ ID NO:1.

6. The composition of claim 4 wherein the protein coding region comprises SEQ ID NO:2.

7. The composition of claim 1, wherein said DNA sequence comprises pWRG-SEO-M.

8. The composition of claim 1, wherein said DNA sequence comprises pWRG-SEO-S.

9. A method for inducing a protective immune response to a hantavirus protein in a mammal, comprising (i) preparing a nucleic acid encoding a determinant of a hantavirus protein operatively linked to a promoter operative in cells of a mammal; (ii) coating the nucleic acid in (i) onto carrier particles; (iii) accelerating the coated carrier particles into epidermal cells of the mammal in vivo; and (iv) detecting a protective immune response in said mammal upon exposure to a hantavirus.

10. The method according to claim 9 wherein the carrier particles are gold.

11. The method according to claim 9 wherein the protein determinant is chosen from the group consisting of M genome segment proteins and S

12. The method according to claim 9 wherein said hantavirus is chosen from the group consisting of Seoul virus, Dobrava virus, Puumala virus, Hantaan virus, Sin Nombre virus, Black Creek Canal virus, Bayou virus, New York virus, Andes virus, and Laguna Negra virus.

13. The method of claim 12 wherein said hantavirus is Seoul virus.

14. The method according to claim 13 wherein said nucleic acid comprises SEQ ID NO:1.

15. The method according to claim 13 wherein said nucleic acid comprises SEQ ID NO:2.

16. The method according to claim 13 wherein said nucleic acid comprises SEQ ID NO: 1 and SEQ ID NO:2.

17. A method for inducing a protective immune response to a hantavirus infection in a mammal comprising (i) preparing a nucleic acid encoding a determinant of a first hantavirus protein operatively linked to a promoter operative in cells of a mammal; (ii) coating the nucleic acid in (i) onto carrier particles; (iii) accelerating the coated carrier particles into epidermal cells of the mammal in vivo; and (iv) detecting an immune response in said mammal upon a exposure to a second hantavirus.

18. The method according to claim 17 wherein said first hantavirus is SEOV.

19. The method according to claim 18 wherein said second hantavirus is Dobrava virus.

20. The method according to claim 18 wherein said second hantavirus is Hantaan virus.

21. The method according to claim 16 wherein said nucleic acid is selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:2.

22. A vaccine against hantavirus infection comprising the composition of claim 3.

23. A vaccine against hantavirus infection comprising the composition of claim 4.

24. A vaccine against hantavirus infection comprising the composition of claim 5.

25. A vaccine against hantavirus infection comprising the composition of claim 6.

26. A multivalent vaccine for protection against infection with more than one hantavirus comprising a composition of matter comprising a carrier particle having one or more DNA sequence coated onto the carrier particle, the DNA sequence comprising a promoter operative in the cells of a mammal and a protein coding region coding for a determinant of a first hantavirus protein said hantavirus selected from the group consisting of SEOV, Dobrava, Puumala, Hantaan, Sin Nombre virus, Black Creek Canal virus, Bayou virus, New York virus, Andes virus, and Laguna Negra virus.

27. The multivalent vaccine of claim 26, further comprising a composition comprising a carrier particle having one or more DNA sequence coated onto the carrier particle, the DNA sequence comprising a promoter operative in the cells of a mammal and a protein coding region coding for a determinant of a second hantavirus different from said first hantavirus, said second hantavirus selected from the group consisting of Seoul virus, Dobrava virus, Puumala virus, Hantaan virus, Sin Nombre virus, Black Creek Canal virus, Bayou virus, New York virus, Andes virus, and Laguna Negra virus.

L12 ANSWER 8 OF 12 USPTAFULL on STN

2002:16571 Prophylactic and therapeutic monoclonal antibodies.

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**Schmaljohn, Connie S.**, Frederick, MD, UNITED STATES

US 2002009447 A1 20020124

APPLICATION: US 2001-781124 A1 20010209 (9)

PRIORITY: US 2000-182066P 20000211 (60)

DOCUMENT TYPE: Utility; APPLICATION.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB In this application are described vaccinia monoclonal antibodies. Also

CLM

methods of using individual antibodies or mixtures thereof for the detection, prevention, and/or therapeutical treatment of vaccinia virus infections in vitro and in vivo.

What is claimed is:

1. A composition comprising one or more monoclonal antibody directed against a vaccinia virus antigen.
2. The composition of claim 1 wherein said vaccinia virus antigen is L1R.
3. The composition of claim 1 wherein said vaccinia virus antigen is A33R.
4. The composition of claim 2 wherein said composition further comprises one or more monoclonal antibody directed against vaccinia A33R.
5. The composition of claim 4 wherein said composition further comprises one or more monoclonal antibody directed against an antigen chosen from the group consisting essentially of: vaccinia H3L, D8L, B5R, A27L and A17L.
6. The composition of claim 4 wherein said composition inhibits vaccinia virus infection in a subject in vivo.
7. The composition of claim 6 wherein said subject is avian or mammalian.
8. The composition of claim 4 wherein said composition ameliorates symptoms of vaccinia virus infection when said composition is administered to a subject after infection with vaccinia virus.
9. The composition of claim 8 wherein said subject is avian or mammalian.
10. The composition of claim 2 wherein said monoclonal antibody immunoprecipitates L1R in vitro.
11. The composition of claim 3 wherein said monoclonal antibody immunoprecipitates A33R in vitro.
12. A therapeutic composition for ameliorating symptoms of vaccinia virus infection comprising the composition of claim 4, and a pharmaceutically acceptable excipient.
13. A passive vaccine against vaccinia virus infection comprising the composition of claim 4.
14. An anti-vaccinia composition, comprising one or more monoclonal antibodies, wherein at least two of said monoclonal antibodies are directed against L1R and A33R, in an amount effective for inhibiting vaccinia virus infection, and a pharmaceutically acceptable carrier.
15. A method of treating vaccinia virus infection comprising administering to a patient in need of said treatment an effective amount of a composition according to claim 4.
16. The composition according to claim 1 wherein said vaccinia virus antigen is chosen from the vaccinia strain Connaught, IHD-J, Brighton, WR, Lister, Copenhagen, Ankara, Dairen I, L-IPV, LC16M8, LC16MO, L1VP, Tian Tan, WR 65-16, Wyeth.
17. A poxvirus monoclonal antibody composition comprising monoclonal antibodies against a homolog of a vaccinia antigen chosen from the group consisting of L1R and A33R, said poxvirus chosen from the group consisting of: orthopoxvirus, parapoxvirus, avipoxvirus, capripoxvirus, leporipoxvirus, suipoxvirus, molluscimpoxvirus, and yatapoxvirus.

L12 ANSWER 9 OF 12 USPATFULL on STN

2001:107871 DNA vaccines against tick-borne flaviviruses.

**Schmaljohn, Connie S.**, Frederick, MD, United States

The United States of America as represented by the Secretary of the Army, Washington, DC, United States (U.S. corporation)

US 6258788 B1 20010710

APPLICATION: US 1998-197218 19981120 (9)

PRIORITY: US 1997-65750P 19971120 (60)

DOCUMENT TYPE: Utility; GRANTED.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Particle mediated immunization of tick-borne flavivirus genes confers homologous and heterologous protection against tick borne encephalitis.

CLM What is claimed is:

1. A method for inducing a protective immune response to a tick-borne

encoding an antigenic determinant of a tick-borne flavivirus prM/E protein operatively linked to a CMV promoter operative in cells of a mammal, which nucleic acid is suitable for stably producing the antigenic determinant in a mammal; (ii) coating the nucleic acid in (i) onto carrier particles; (iii) accelerating the coated carrier particles into epidermal cells of the mammal in vivo; and (iv) inducing a protective immune response in said mammal upon exposure to a tick-borne flavivirus.

2. The method according to claim 1 wherein the carrier particles are gold.

3. The method according to claim 1 wherein the tick-borne flavivirus prM/E protein is selected from the group consisting of Russian spring summer encephalitis prM/E proteins, and Central European encephalitis prM/E proteins.

4. The method according to claim 1 wherein the nucleic acid encodes a protein coding region comprising SEQ ID NO:1.

5. A method for inducing a protective immune response to a tick-borne flavivirus protein in a mammal, comprising (i) preparing a nucleic acid encoding an antigenic determinant of a Russian spring summer encephalitis tick-borne flavivirus prM/E protein operatively linked to a promoter operative in cells of a mammal, which nucleic acid encodes a protein coding region comprising SEQ ID NO:2 and is suitable for stably producing the antigenic determinant in a mammal; (ii) coating the nucleic acid in (i) onto carrier particles; (iii) accelerating the coated carrier particles into epidermal cells of the mammal in vivo; and (iv) inducing a protective immune response in said mammal upon exposure to a tick-borne flavivirus.

6. The method according to claim 1 wherein the nucleic acid encodes a protein coding region comprising SEQ ID NO: 1 and SEQ ID NO:2.

7. A kit for inducing a protective immune response to a tick-borne flavivirus protein in a mammal, comprising packaged in association: (a) a nucleic acid encoding an antigenic determinant of a tick-borne flavivirus prM/E protein operatively linked to a CMV promoter operative in cells of a mammal, which nucleic acid is suitable for stably producing the antigenic determinant in a mammal; (b) one or both of a coating solution and/or components of a coating solution; and (c) carrier particles.

8. The kit of claim 7, wherein the tick-borne flavivirus prM/E protein is selected from the group consisting of Russian spring summer encephalitis prM/E proteins, and Central European encephalitis prM/E proteins.

9. The kit of claim 7, wherein the nucleic acid encodes a protein coding region comprising SEQ ID NO:1.

10. The kit of claim 7, wherein the nucleic acid encodes a protein coding region comprising SEQ ID NO:1 and SEQ ID NO:2.

11. A kit for inducing a protective immune response to a tick-borne flavivirus protein in a mammal, comprising packaged in association: (a) a nucleic acid encoding an antigenic determinant of a Russian spring summer encephalitis tick-borne flavivirus prM/E protein operatively linked to a promoter operative in cells of a mammal which nucleic acid encodes a protein coding region comprising SEQ ID NO:2 and is suitable for stably producing the antigenic determinant in a mammal; (b) one or both of a coating solution and/or components of a coating solution; and (c) carrier particles.

L12 ANSWER 10 OF 12 USPATFULL on STN

2001:36806 Genetic induction of anti-viral immune response and genetic vaccine for filovirus.

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Fuller, Deborah L., Oregon, WI, United States

Schmaljohn, Alan, Frederick, MD, United States

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US 6200959 B1 20010313

APPLICATION: US 1996-760615 19961204 (8)

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB An approach to genetic vaccine methodology is described. A genetic construction encoding antigenic determinants of a filovirus is transfected into cells of the vaccinated individuals using a particle

CLM

cells to produce an immune response to those antigens.

What is claimed is:

1. A method of inducing an immune response to a Marburg or Ebola virus glycoprotein in a mammal, said method comprising: (a) providing a genetic construction comprising a promoter operative in cells of the mammal and a coding region for a determinant of the glycoprotein, the genetic construction not comprising sequences necessary for replication of the virus; (b) coating copies of the genetic construction onto carrier particles small in size in relation to the size of the cells of the mammal; and (c) accelerating the coated carrier particles into epidermal cells of the mammal in vivo, thereby inducing an immune response against the glycoprotein.
2. A method as claimed in claim 1 wherein the carrier particles are accelerated by a gaseous pulse in order to accelerate the carrier particles toward the mammal.
3. A method as claimed in claim 1 wherein the protein coding region encodes a glycoprotein selected from the group consisting of Ebola Zaire virus gp125, Marburg Musoke virus gp170, and Marburg Ravn virus glycoprotein.
4. A method as claimed in claim 1 wherein the protein coding region comprises SEQ ID NO: 1.
5. A method as claimed in claim 1 wherein the protein coding region comprises SEQ ID NO: 3.
6. A method as claimed in claim 1 wherein the protein coding region comprises SEQ ID NO: 5.
7. A composition of matter comprising a carrier particle and a genetic construction coated onto the carrier particle, wherein the genetic construction comprises a promoter operative in the cells of a mammal and a coding region for a determinant of a Marburg or Ebola virus glycoprotein.
8. A composition as claimed in claim 7 wherein the protein coding region encodes a glycoprotein selected from the group consisting of Ebola Zaire virus gp125, Marburg Musoke virus gp170, and Marburg Ravn virus glycoprotein.
9. A composition as claimed in claim 7 wherein the protein coding region comprises SEQ ID NO: 1.
10. A composition as claimed in claim 7 wherein the protein coding region comprises SEQ ID NO: 3.
11. A composition as claimed in claim 7 wherein the protein coding region comprises SEQ ID No. 5.

L12 ANSWER 11 OF 12 USPATFULL on STN

97:24716 Hantavirus vaccine.

**Schmaljohn, Connie S.**, Frederick, MD, United States

McClain, David J., Frederick, MD, United States

Dalrymple, deceased, Joel, late of Myersville, MD, United States by Lonnie

Dalrymple, Legal Representative

The United States of America as represented by the Secretary of the Army,

Washington, DC, United States (U.S. government)

US 5614193 19970325

APPLICATION: US 1994-218943 19940328 (8)

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Vaccine formulations for inducing protective immune response to Hantaviruses in humans are disclosed. These formulations include an attenuated vaccinia virus vector containing cDNA's encoding Hantavirus nucleocapsid N protein, G1 and G2 glycoproteins. Methods for the use of these formulations also are disclosed.

CLM

What is claimed is:

1. A vaccine formulation suitable for use in a human, comprising: (i) infectious vaccinia virus that comprises a DNA molecule encoding (a) the Hantaan virus polypeptides designated N, G1 and G2, and (b) all vaccinia virus polypeptides necessary for replication of said virus in a cell derived from said human, but not a functional thymidine kinase; and (ii) a pharmaceutically-acceptable carrier, excipient or diluent, wherein said vaccine formulation comprises a single dose of  $5 \times 10^5$  to  $7 \times 10^7$  plaque forming units of vaccinia virus.
2. The vaccine formulation according to claim 1, wherein the nucleotide sequence encoding said G1 polypeptide and said G2 polypeptide is operably linked to the vaccinia virus 7.5 kD promoter, and wherein the

the vaccinia virus 11 kD promoter.

3. The vaccine formulation according to claim 1, wherein said G1 has the sequence (SEQ ID NO: 1): ##STR6## said G2 has the sequence (SEQ ID NO: 2): ##STR7## and said N has the sequence (SEQ ID NO: 3): ##STR8##

4. The vaccine formulation according to claim 3, wherein said DNA molecule comprises a wild-type vaccinia virus genome with the cDNA of the M and S genomic segments of the Hantaan serotype inserted in the vaccinia virus thymidine kinase coding region.

5. The vaccine formulation according to claim 4, wherein said cDNA comprises the sequence (SEQ ID NO: 4): ##STR9## and (SEQ ID NO: 5): ##STR10##

6. The vaccine formulation according to claim 1, wherein said single dose comprises  $5 \times 10^5$  to  $7 \times 10^7$  plaque-forming units.

7. The vaccine formulation according to claim 1, wherein said single dose comprises  $5 \times 10^5$  to  $1 \times 10^6$  plaque-forming units.

8. The vaccine formulation according to claim 1, wherein said single dose comprises  $5 \times 10^5$  to plaque-forming units.

9. The vaccine formulation according to claim 1, wherein said single dose comprises  $3.4 \times 10^7$  plaque-forming units.

10. The vaccine formulation according to claim 9, wherein said pharmaceutically-acceptable carrier, excipient or diluent further comprises lactose and human serum albumin.

11. The vaccine formulation according to claim 10, wherein said lactose is 5% (w/v) of said formulation and said human serum albumin is 1% (w/v) of said formulation.

12. The vaccine formulation according to claim 11, wherein said formulation further comprises neomycin of no more than 25 µg per single dose of said formulation.

13. The vaccine formulation according to claim 12, wherein said single dose of said formulation is in a volume of 0.1 to 1.0 ml.

14. The vaccine formulation according to claim 1, wherein said formulation is in a form suitable for a route of administration selected from the group consisting of subcutaneous, intramuscular and intradermal.

15. A method for inducing a Hantavirus-protective immune response in a human, comprising the steps of: (i) providing a vaccine formulation suitable for use in a human comprising (a) infectious vaccinia virus activity and comprising a DNA molecule encoding (1) the Hantaan virus polypeptides designated N, G1 and G2, and (2) all vaccinia virus polypeptides necessary for replication of said virus in a cell derived from said human, but not a functional thymidine kinase; and (b) a pharmaceutically-acceptable carrier, excipient or diluent, and (ii) administering said vaccine formulation to said human, wherein a single dose of said vaccine formulation comprises  $5 \times 10^5$  to  $7 \times 10^7$  plaque forming units of vaccinia virus.

16. The method according to claim 15, wherein the nucleotide sequence encoding said G1 polypeptide and said G2 polypeptide is operably linked to the vaccinia virus 7.5 kD promoter, and wherein the nucleotide sequence encoding said N polypeptide is operably linked to the vaccinia virus 11 kD promoter.

17. The method according to claim 16, wherein said G1 has the sequence (SEQ ID NO: 1): ##STR11## said G2 has the sequence (SEQ ID NO: 2): ##STR12## and said N has the sequence (SEQ ID NO: 3): ##STR13##

18. The method according to claim 17, wherein said DNA molecule comprises a wild-type vaccinia virus genome with the cDNA of the M and S genomic segments of the Hantaan serotype inserted in the vaccinia virus thymidine kinase coding region.

19. The method according to claim 18, wherein said cDNA comprises the sequence: (SEQ ID NO: 4): ##STR14## and (SEQ ID NO: 5): ##STR15##

20. The method according to claim 15, wherein said single dose comprises  $5 \times 10^5$  to  $7 \times 10^7$  plaque-forming units.

5×10<sup>5</sup> to 1×10<sup>6</sup> plaque-forming units.

22. The method according to claim 15, wherein said single dose comprises 5×10<sup>5</sup> plaque-forming units.

23. The method according to claim 15, wherein said single dose comprises 3.4×10<sup>7</sup> plaque-forming units.

24. The method according to claim 23, wherein said pharmaceutically-acceptable carrier, excipient or diluent further comprises lactose and human serum albumin.

25. The method according to claim 24, wherein said lactose is 5% (w/v) of said formulation and said human serum albumin is 1% (w/v) of said formulation.

26. The method according to claim 25, wherein said formulation further comprises neomycin of no more than 25 µg per single dose of said formulation.

27. The method according to claim 26, wherein said single dose of said formulation is in a volume of 0.1 to 1.0 ml.

28. The method according to claim 27, wherein said administering is by a route selected from the group consisting of subcutaneous, intramuscular and intradermal.

L12 ANSWER 12 OF 12 USPATFULL on STN

94:26451 Nucleotide sequences encoding the expression of a Hantaan virus nucleocapsid protein and G1 and G2 glycoproteins.

Dalrymple, Joel M., Myersville, MD, United States

Schmaljohn, Connie S., Frederick, MD, United States

The United States of America as represented by the Secretary of the Army, Washington, DC, United States (U.S. government)

US 5298423 19940329

APPLICATION: US 1991-799479 19911114 (7)

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB Nucleotide sequences coding for Hantaan virus nucleocapsid protein and glycoproteins G1 and G2 can be used to produce these proteins for vaccine and diagnostic applications.

CLM What is claimed is:

1. A vector comprising a nucleotide molecule selected from the group consisting of a nucleotide molecule coding for a Hantaan virus nucleocapsid N protein and a nucleotide molecule coding for a precursor of Hantaan virus G1 and G2 glycoproteins.

2. A vector as claimed in claim 1, wherein said nucleotide molecule coding for said nucleocapsid N protein is defined by the formula: ##STR1##

3. A vector as claimed in claim 2, wherein said nucleotide molecule coding for said nucleocapsid N protein further comprises at least one of (a) a leader-sequence that precedes said nucleotide molecule coding for said nucleocapsid N protein, wherein said leader-sequence comprises a sequence defined by the formula: TAGTAGTAGACTCCCTAAAGAGCTACTAGAACACG, and (b) a tail-sequence that follows said nucleotide molecule coding for said nucleocapsid N protein, wherein said tail-sequence comprises a sequence defined by the formula: ##STR2##

4. A vector as claimed in claim 1, wherein said nucleotide molecule coding for said precursor is defined by the formula: ##STR3##

5. A vector as claimed in claim 4, wherein said nucleotide molecule coding for said precursor further comprises at least one of (a) a leader-sequence that precedes said nucleotide molecule coding for said precursor, wherein said leader-sequence is comprised of a sequence defined by the formula: ##STR4## (b) a tail-sequence that follows said nucleotide molecule coding for said precursor, wherein said tail-sequence is comprised of a sequence defined by the formula: ##STR5##

6. A vector as claimed in claim 1, wherein said nucleocapsid N protein has an amino acid sequence defined by the formula: ##STR6##

7. A vector as claimed in claim 1, wherein said precursor has an amino acid sequence defined by the formula: ##STR7##

8. A vector as claimed in claim 7, wherein said amino acid sequence is preceded by a leader-sequence that comprises a sequence defined by the formula: ##STR8##



9. A cDNA molecule comprising a nucleotide molecule selected from the group consisting of a nucleotide molecule coding for a Hantaan virus nucleocapsid N protein and a nucleotide molecule coding for a precursor of Hantaan virus G1 and G1 glycoproteins.

10. A cDNA molecule as claimed in claim 9, wherein said nucleotide molecule coding for said nucleocapsid N protein is defined by the formula: ##STR9##

11. A cDNA molecule as claimed in claim 10, wherein said nucleotide molecule coding for said nucleocapsid N protein further comprises at least one of (a) a leader-sequence that precedes said nucleotide molecule coding for said nucleocapsid N protein, wherein said leader-sequence is comprised of a sequence defined by the formula: TAGTAGTAGACTCCCTAAAGAGCTACTAGAACAACG, and (b) a tail-sequence that follows said nucleotide molecule coding for said nucleocapsid N protein, wherein said tail-sequence is comprised of a sequence defined by the formula: ##STR10##

12. A cDNA molecule as claimed in claim 9, wherein said nucleotide molecule coding for said precursor is defined by the formula: ##STR11##

13. A cDNA molecule as claimed in claim 12, wherein said nucleotide molecule coding for said precursor further comprises at least one of (a) a leader-sequence that precedes said nucleotide molecule coding for said precursor, wherein said leader-sequence is comprised of a sequence defined by the formula: ##STR12## (b) a tail-sequence that follows said nucleotide molecule coding for said precursor, wherein said tail-sequence is comprised of a sequence defined by the formula: ##STR13##

=> d his

(FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006)

FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

```
      E CHANG G J J/IN
      E CHANG GWONG J J/IN
L1      2 S E4
      E KONISHI E/AU
      E KONISHI E/IN
L2      24958 S CMV OR CMV-IE
L3      3918 S L2 AND KOZAK
L4      3136 S L3 AND TERMINATION
L5      0 S L4 AND (POLY W A)
L6      760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR
L7      33 S L6 AND CMV/CLM
L8      11 S L7 AND AY<1999
L9      498 S L6 AND PCDNA3?
L10     9 S L9 AND AY<1999
L11     3 S L10 NOT L8
      E SCHMALJOHN C S/IN
L12     12 S E4
```

=> s (flavivir? or dengue or japanese encephalitis or yellow fever vir?)

```
      2969 FLAVIVIR?
      2750 DENGUE
      436101 JAPANESE
      8576 ENCEPHALITIS
      1714 JAPANESE ENCEPHALITIS
      (JAPANESE(W)ENCEPHALITIS)
      259856 YELLOW
      22833 FEVER
      724613 VIR?
      1392 YELLOW FEVER VIR?
      (YELLOW(W)FEVER(W)VIR?)
L13     4840 (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELLOW FEVER
      VIR?)
```

=> s l13 and (PrM? and E)

```
      5436 PRM?
      2829024 E
L14     161 L13 AND (PRM? AND E)
```

=> s l14 and (signal sequence)

```
      1361043 SIGNAL
      790269 SEQUENCE
      29349 SIGNAL SEQUENCE
      (SIGNAL(W)SEQUENCE)
L15     82 L14 AND (SIGNAL SEQUENCE)
```

=> s 115 and ay<1999  
2811963 AY<1999  
L16 15 L15 AND AY<1999

=> d 116,cbib,ab,clm,1-15

L16 ANSWER 1 OF 15 USPATFULL on STN

2005:283181 Chimeric **flavivirus** vaccines.

Chambers, Thomas J., St. Louis, MO, UNITED STATES

Monath, Thomas P., Harvard, MA, UNITED STATES

Guirakhoo, Farshad, Melrose, MA, UNITED STATES

Arroyo, Juan, S. Weymouth, MA, UNITED STATES

Acambis, Inc., Cambridge, MA, UNITED STATES (U.S. corporation) St. Louis

University, St. Louis, MO, UNITED STATES (U.S. corporation)

US 6962708 B1 20051108

**APPLICATION: US 1998-121587 19980723 (9)**

DOCUMENT TYPE: Utility; GRANTED.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A chimeric live, infectious, attenuated virus containing a **yellow fever virus**, in which the nucleotide sequence for a **prM-E** protein is either deleted, truncated, or mutated, so that functional **prM-E** protein is not expressed, and integrated into the genome of the **yellow fever virus**, a nucleotide sequence encoding a **prM-E** protein of a second, different **flavivirus**, so that the **prM-E** protein of the second **flavivirus** is expressed.

CLM What is claimed is:

1. A chimeric live, infectious, attenuated virus, comprising: a **yellow fever virus** in which the nucleotide sequence encoding a **prM-E** protein is either deleted, truncated, or mutated so that functional **yellow fever virus prM-E** protein is not expressed, and integrated into the genome of said **yellow fever virus**, a nucleotide sequence encoding a **prM-E** protein of a second, different **flavivirus**, to that said **prM-E** protein of said second **flavivirus** is expressed, wherein the capsid protein of said chimeric virus is from **yellow fever virus**.
2. The chimeric virus of claim 1, wherein said second **flavivirus** is a **Japanese Encephalitis (JE)** virus.
3. The chimeric virus of claim 1, wherein the nucleotide sequence encoding the **prM-E** protein of said, second, different **flavivirus** replaces the nucleotide sequence encoding the **prM-E** protein of said **yellow fever virus**.
4. The chimeric virus of claim 1, wherein said nucleotide sequence encoding said **prM-E** protein of said second, different **flavivirus** comprises a mutation that prevents **prM** cleavage to produce M protein.
5. The chimeric virus of claim 1, wherein the NS2B-3-protease recognition site and the signal sequences and cleavage sites at the C/**prM** and **E**/NS1 junctions are maintained in construction of said chimeric virus.
6. The chimeric virus of claim 1, wherein said second **flavivirus** is a Murray Valley Encephalitis virus.
7. The chimeric virus of claim 1, wherein said second **flavivirus** is a St. Louis Encephalitis virus.
8. The chimeric virus of claim 1, wherein said second **flavivirus** is a West Nile virus.
9. The chimeric virus of claim 1, wherein said second **flavivirus** is a Tick-borne Encephalitis virus.
10. The chimeric virus of claim 1, wherein the **signal sequence** at the C/**prM** junction is maintained in construction of said chimeric virus.
11. A method of preventing or treating **Japanese encephalitis** virus infection in a patient, said method comprising administering to said patient a chimeric, live, infectious, attenuated virus comprising: a **yellow fever virus** in which the nucleotide sequence encoding a **prM-E** protein is either deleted, truncated, or mutated so that functional **yellow fever virus prM-E** protein is not expressed, and integrated into the genome of said **yellow fever virus**, a nucleotide sequence encoding a **prM-E** protein of **Japanese encephalitis** virus strain SA-14-14-2 or **Japanese encephalitis** virus strain Nakayama, wherein the capsid protein of said chimeric virus is from **yellow fever virus**.
12. The method of claim 11, wherein the nucleotide sequence encoding the **prM-E** protein of said **Japanese encephalitis** virus replaces the

fever virus.

13. The method of claim 11, wherein said nucleotide sequence encoding said **prM-E** protein of said **Japanese encephalitis** virus comprises a mutation that prevents **prM** cleavage to produce M protein.

14. The method of claim 11, wherein the NS2B-3 protease recognition site and the signal sequences and cleavage sites at the C/**prM** and **E**/NS1 junctions are maintained in connection of said chimeric virus.

L16 ANSWER 2 OF 15 USPTAFULL on STN

2002:167888 Recombinant nonstructural protein subunit vaccine against **flaviviral** infection.

McDonnell, Michael, Kailua, HI, United States

Peters, Iain, Honolulu, HI, United States

Coller, Beth-Ann, Aiea, HI, United States

Hawaii Biotechnology Group, Inc., Aiea, HI, United States (U.S. corporation)

US 6416763 B1 20020709

**APPLICATION: US 1998-143077 19980828 (9)**

DOCUMENT TYPE: Utility; GRANTED.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The recombinant expression and secretion from eucaryotic host cells, particularly *Drosophila* cells, of **Flavivirus** nonstructural (NS) protein, particularly NS1, is useful in combination with **Flavivirus** truncated envelope (**E**) protein to protect a host subject from infection and disease from **Flavivirus** species. Further, NS1 is useful as a diagnostic of **flaviviral** infection.

Compositions of truncated **flaviviral** envelope protein and **flaviviral** nonstructural protein induce high titer virus neutralizing antibodies believed to be important in protection against **flaviviral** infection and which are useful in diagnosis of infection by the virus.

CLM What is claimed is:

1. An immunogenic composition which induces an immunological response in a host subject inoculated with said composition comprising a carrier and a mixture comprising a **Flavivirus** truncated envelope (**E**) protein and a **Flavivirus** nonstructural (NS) protein, wherein said nonstructural protein (NS) protein has been secreted as a recombinantly produced protein, from *Drosophila* cells, and wherein the truncated envelope (**E**) protein comprises approximately 80%**E**, wherein said 80%**E** represents a portion of the envelope protein that comprises approximately 80% of its length starting from amino acid 1 at its N-terminus.

2. The immunogenic composition of claim 1, wherein said envelope protein (**E**) protein has been secreted as a recombinantly produced protein from *Drosophila* cells.

3. The immunogenic composition of claim 1 wherein the non-structural (NS) protein is encoded in a DNA construct operably linked downstream from human tissue plasminogen activator prepropeptide secretion leader (tPA<sub>L</sub>).

4. The immunogenic composition of claim 1 wherein said **Flavivirus** is a **dengue** virus.

5. The immunogenic composition of claim 1 wherein said *Drosophila* cells are *D. melanogaster* Schneider cells.

6. The immunogenic composition of claim 2 wherein said *Drosophila* cells are *D. melanogaster* Schneider cells.

7. A method to produce an immunogenic composition comprising (a) culturing the *Drosophila* cells modified to contain a DNA molecule which comprises a nucleotide sequence encoding a nonstructural (NS) protein of the **Flavivirus** against which enhanced protection is sought in culture medium under conditions favorable for expression of the encoding nucleotide sequence so that the cells secrete said nonstructural (NS) protein of the **Flavivirus** strain against which enhanced protection is sought; (b) recovering the nonstructural (NS) protein from the culture medium; and (c) combining said NS with a **Flavivirus** truncated envelope protein, wherein the truncated envelope (**E**) protein comprises approximately 80%**E**, wherein said 80%**E** represents a portion of the envelope protein that comprises approximately 80% of its length starting from amino acid 1 at its N-terminus.

8. A method to produce an immunogenic composition comprising (a) culturing the *Drosophila* cells modified to contain a DNA molecule which comprises a nucleotide sequence encoding a nonstructural (NS1) protein of the **Flavivirus** against which enhanced protection is sought in culture medium under conditions favorable for expression of the encoding

**Flavivirus** strain against which enhanced protection is sought; (b) recovering the NS1 protein from the culture medium; and (c) combining said NS1 with a **Flavivirus** truncated envelope protein, wherein the truncated envelope (E) protein comprises approximately 80%E, wherein said 80%E represents a portion of the envelope protein that comprises approximately 80% of its length starting from amino acid 1 at its N-terminus.

9. The immunogenic composition of claim 1, wherein the nonstructural (NS) protein is NS1.

10. An immunodiagnostic for the detection of a **Flavivirus**, wherein said immunodiagnostic comprises, the immunogenic composition of claim 1.

11. The immunodiagnostic of claim 10, wherein the nonstructural (NS) protein is NS1.

12. The immunodiagnostic of claim 10, wherein said **Flavivirus** is a **dengue** virus.

13. The immunodiagnostic of claim 10, wherein said envelope (E) protein has been secreted as a recombinantly produced protein from *Drosophila* cells.

14. The immunodiagnostic of claim 13, wherein said *Drosophila* cells are *D. melanogaster* Schneider cells.

L16 ANSWER 3 OF 15 USPTAFULL on STN

2001:14256 Two-step immunization procedure against the paramyxoviridae family of viruses using recombinant virus and subunit protein preparation.

Klein, Michel H., Willowdale, Canada

Tartaglia, James, Schenectady, NY, United States

Cates, George A., Richmond Hill, Canada

Ewasyshyn, Mary E., Willowdale, Canada

Virogeneitics Corporation, Troy, NY, United States (U.S.

corporation)Connaught Laboratories Limited, North York, Canada (non-U.S. corporation)

US 6180398 B1 20010130

**APPLICATION: US 1996-679065 19960712 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB An immunization strategy to provide protection against disease caused by infection with a paramyxoviridae virus, specifically respiratory syncytial virus (RSV) and parainfluenza virus, is described. A priming intranasal administration of a recombinant virus expressing at least one RSV or PIV protein or immunogenic sequence there first is made to the host followed by a booster administration of at least one purified RSV or PIV protein or immunogenic fragment thereof, which may be adjuvanted with alum. This immunization strategy provides a safe and effective means of controlling RSV and PIV infections. The strategy leads to a stronger protective immune response than other strategies and to the induction of a more balanced Th-1/Th-2 type response than previously attained. Novel recombinant poxviruses are provided containing nucleic acid encoding a paramyxovirus protein or immunogenic fragment thereof is a non-essential region of the poxvirus genome, specifically NYVAC-F and ALVAC-F, which produce the F glycoprotein of RSV.

CLM What is claimed is:

1. A method of inducing an immune response in a host against disease caused by respiratory syncytial virus (RSV), which comprises: initially administering to the host an immunoeffective amount of a recombinant virus vector expressing at least one RSV protein or immunogenic fragment thereof; and subsequently administering to the host an immunoeffective amount of at least one purified RSV protein or immunogenic fragment thereof to achieve a RSV specific immune response in the host.

2. The method of claim 1 wherein said immune response in the host includes the production of virus specific neutralizing antibodies and/or virus specific cytotoxic T-cell responses.

3. The method of claim 2 wherein said recombinant virus is a recombinant pox virus.

4. The method of claim 2 wherein said recombinant virus expresses at least one RSV protein or immunogenic fragment thereof selected from the group consisting of the fusion (F), attachment (G) and matrix (M) proteins.

5. The method of claim 2 wherein said at least one purified RSV protein or immunogenic fragment thereof is selected from the group consisting of the fusion (F), attachment (G) and matrix (M) proteins.

pox virus.

7. The method of claim 5 wherein said recombinant virus expresses at least one RSV protein or immunogenic fragment thereof selected from the group consisting of the fusion (F), attachment (G) and matrix (M) proteins.

8. The method of claim 1 wherein the at least one purified RSV protein or immunogenic fragment thereof is administered with an adjuvant.

9. The method of claim 8 wherein the adjuvant is alum.

L16 ANSWER 4 OF 15 USPATFULL on STN

2000:174106 Subunit immunogenic composition against **dengue** infection.

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Nakano, Eilen, Hon., HI, United States

Clements, David, Honolulu, HI, United States

Hawaii Biotechnology Group, Inc., Aiea, HI, United States (U.S. corporation)

US 6165477 20001226

APPLICATION: US 1997-915152 19970820 (8)

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The **Flaviviridae** comprise a number of medically important pathogens that cause significant morbidity in humans including the **dengue** (DEN) virus, **Japanese encephalitis** (JE) virus, tick-borne encephalitis virus (TBE), and **yellow fever virus** (YF). **Flaviviruses** are generally transmitted to vertebrates by chronically infected mosquito or tick vectors. The viral particle which is enveloped by host cell membranes, comprises a single positive strand genomic RNA and the structural capsid (CA), membrane (M), and envelope (E) proteins. The E and M proteins are found on the surface of the virion where they are anchored in the membrane. Mature E is glycosylated and contains functional domains responsible for cell surface attachment and intraendosomal fusion activities. Problems have arisen in the art with respect to producing recombinant forms of the E glycoprotein that retain their native configuration and attendant properties associated therewith (i.e., ability to induce neutralizing antibody responses). To date, recombinantly produced E glycoproteins have suffered from a number of limitations including improper glycosylation, folding, and disulfide bond formation. The claimed invention has addressed these concerns by providing secreted recombinant forms of the E glycoprotein that are highly immunogenic and appear to retain their native configuration. Carboxy-terminally truncated forms of E containing the amino terminal 395 amino acids and a suitable secretion **signal sequence** were generated in *Drosophila melanogaster* Schneider cell lines. Immunogenic compositions comprising these recombinant envelope glycoproteins were capable of inducing protective, neutralizing antibody responses when administered to a suitable host.

CLM What is claimed is:

1. An immunogenic composition which generates protective, neutralizing antibody responses to a **Flavivirus** in a murine host which responses confer protection against intracerebral challenge by the homologous **Flavivirus**, said strain of **Flavivirus** selected from the group consisting of a strain of **dengue**, a strain of **Japanese encephalitis** virus (JEV), a strain of **yellow fever virus** (YF), and a strain of tick-borne encephalitis virus (TBE) which composition contains an adjuvant; and a portion of the envelope protein (E) of the **Flavivirus** strain against which said responses are sought, which portion is 80% E, wherein said 80% E represents that portion of the envelope protein that constitutes 80% of its length starting from amino acid 1 at its N-terminus and which portion has been secreted as a recombinantly produced protein from *Drosophila* cells.
2. The immunogenic composition of claim 1 wherein said *Drosophila* cells are *D. melanogaster* Schneider cells.
3. The immunogenic composition of claim 1 wherein said adjuvant is an alum adjuvant.
4. An immunogenic composition which generates a neutralizing antibody response to a **Flavivirus** in a murine host against the homologous **Flavivirus**, said strain of **Flavivirus** selected from the group consisting of a strain of **dengue**, a strain of **Japanese encephalitis** virus (JEV), a strain of **yellow fever virus** (YF), and a strain of tick-borne encephalitis virus (TBE) which composition contains an adjuvant; and a portion of the envelope protein (E) of the **Flavivirus** strain against which generation of said response is sought, which portion is 80% E, wherein said 80% E represents that portion of the envelope protein that constitutes 80% of its length starting from amino acid 1 at its N-terminus, and which portion has been secreted as a

5. The immunogenic composition of claim 4 wherein said *Drosophila* cells are Schneider cells.
6. The immunogenic composition of claim 4 wherein said **Flavivirus** is a **dengue** virus.
7. The immunogenic composition of claim 4 wherein the 80% **E** is encoded in a DNA construct operably linked downstream from human tissue plasminogen activator prepropeptide secretion leader (tPA<sub>L</sub>).
8. The immunogenic composition of claim 4 wherein the adjuvant is an alum adjuvant.
9. A method to generate a neutralizing antibody response in a non-human subject against a **Flavivirus** strain, said strain selected from the group consisting of a strain of **dengue**, a strain of YF, a strain of JEV, and a strain of TBE, which method comprises administering to a non-human subject in need of generating said response an effective amount of the immunogenic composition of claim 4.
10. The method of claim 9 wherein said **Flavivirus** is a **dengue** virus.
11. The immunogenic composition of claim 1 wherein the 80% **E** is encoded in a DNA construct operably linked downstream from a human tissue plasminogen activator prepropeptide secretion leader (tPA<sub>L</sub>) sequence.
12. The immunogenic composition of claim 1 wherein said **Flavivirus** is a **dengue** virus.
13. The immunogenic composition of claim 2 wherein the **Flavivirus** is a **dengue** virus.

L16 ANSWER 5 OF 15 USPTAFULL on STN

2000:142128 Methods of preparing carboxy-terminally truncated recombinant **flavivirus** envelope glycoproteins employing *drosophila melanogaster* expression systems.

Ivy, John, Kailua, HI, United States

Nakano, Eilen, Honolulu, HI, United States

Clements, David, Honolulu, HI, United States

Hawaii Biotechnology Group, Inc., Aiea, HI, United States (U.S. corporation)

US 6136561 20001024

**APPLICATION: US 1997-937195 19970925 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The **Flaviviridae** comprise a number of medically important pathogens that cause significant morbidity in humans including the **dengue** (DEN) virus, **Japanese encephalitis** (JE) virus, tick-borne encephalitis virus (TBE), and **yellow fever virus** (YF). **Flaviviruses** are generally transmitted to vertebrates by chronically infected mosquito or tick vectors. The viral particle which is enveloped by host cell membranes, comprises a single positive strand genomic RNA and the structural capsid (CA), membrane (M), and envelope (E) proteins. The E and M proteins are found on the surface of the virion where they are anchored in the membrane. Mature E is glycosylated and contains functional domains responsible for cell surface attachment and intraendosomal fusion activities. Problems have arisen in the art with respect to producing recombinant forms of the E glycoprotein that retain their native configuration and attendant properties associated therewith (i.e., ability to induce neutralizing antibody responses). To date, recombinantly produced E glycoproteins have suffered from a number of limitations including improper glycosylation, folding, and disulfide bond formation. The claimed invention has addressed these concerns by providing secreted recombinant forms of the E glycoprotein that are highly immunogenic and appear to retain their native configuration. Carboxy-terminally truncated forms of E containing the amino terminal 395 amino acids and a suitable secretion **signal sequence** were generated in *Drosophila melanogaster* Schneider cell lines. The recombinant proteins produced by this expression system should prove useful, inter alia, as immunogens and diagnostic reagents.

CLM What is claimed is:

1. An expression system for the recombinant production and secretion of a portion of an envelope (E) protein of a **Flavivirus** selected from the group consisting of **dengue** virus, **Japanese encephalitis** virus (JEV), tick-borne encephalitis virus (TBE) and **yellow fever virus** (YF), which expression system comprises *Drosophila* cells modified to contain a DNA molecule which comprises (a) a first nucleotide sequence encoding said portion of said E protein of the **Flavivirus** strain against which protection is sought, which portion is the N-terminal 80%

nucleotide sequence which encodes a secretory leader sequence or a secretory **signal sequence** operably linked to said first nucleotide sequence and positioned so as to produce a fusion protein when said first and said second nucleotide sequences are expressed in a eucaryotic cell, said encoding sequences operably linked to control sequences capable of effecting expression of said encoding nucleotide sequences in eucaryotic cells.

2. The expression system of claim 1 wherein said secretory leader sequence is human tissue plasminogen activator prepropeptide secretion leader (tPA<sub>L</sub>) and optionally includes the premembrane leader of the **E** protein.

3. A method to produce a portion of an **E** protein of a **Flavivirus** selected from the group consisting of **dengue virus**, **Japanese encephalitis virus** (JEV), tick-borne encephalitis virus (TBE) and **yellow fever virus** (YF), which method comprises (a) culturing the Drosophila cells of claim 1 in culture medium under conditions favorable for expression of the encoding nucleotide sequence so that the cells secrete said portion of the **E** protein of the **Flavivirus** strain against which protection is sought, which portion is the N-terminal 80% of the protein from residue 1 to residue 395 into the medium; and (b) recovering the portion of the **E** protein from the culture medium.

4. A method to produce a portion of an **E** protein of a **Flavivirus** selected from the group consisting of **dengue virus**, **Japanese encephalitis virus** (JEV), tick-borne encephalitis virus (TBE) and **yellow fever virus** (YF), which method comprises (a) culturing the Drosophila cells of claim 2 in culture medium under conditions favorable for expression of the encoding nucleotide sequence so that the cells secrete said portion of the **E** protein of the **Flavivirus** strain against which protection is sought, which portion is the N-terminal 80% of the protein from residue 1 to residue 395 into the medium; and (b) recovering the portion of the **E** protein from the culture medium.

5. The expression system of claim 1 wherein the N-terminal 80% of the **E** protein from residue 1 to residue 395 is **dengue virus E** protein.

6. The method of claim 3 wherein the N-terminal 80% of the **E** protein from residue 1 to residue 395 is **dengue virus E** protein.

7. The method of claim 4 wherein the N-terminal 80% of the **E** protein from residue 1 to residue 395 is **dengue virus E** protein.

8. The expression system of claim 1, wherein the Drosophila cells are Drosophila Schneider cells.

9. The expression system of claim 2, wherein the Drosophila cells are Drosophila Schneider cells.

10. The method of claim 3, wherein the Drosophila cells are Drosophila Schneider cells.

11. The method of claim 4, wherein the Drosophila cells are Drosophila Schneider cells.

12. The expression system of claim 5, wherein the Drosophila cells are Drosophila Schneider cells.

13. The method of claim 6, wherein the Drosophila cells are Drosophila Schneider cells.

14. The method of claim 7, wherein the Drosophila cells are Drosophila Schneider cells.

L16 ANSWER 6 OF 15 USPATFULL on STN

2000:77209 Polynucleotide encoding a promonocyte associated protein.

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Au-Young, Janice, Berkeley, CA, United States

Corley, Neil C., Mountain View, CA, United States

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US 6077693 20000620

**APPLICATION: US 1998-79981 19980514 (9)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The invention provides a human promonocyte associated protein (**PRMNC**) and polynucleotides which identify and encode **PRMNC**. The invention

CLM

antagonists. The invention also provides methods for diagnosing, treating or preventing disorders associated with expression of **PRMNC**. What is claimed is:

1. An isolated and purified polynucleotide comprising a polynucleotide sequence selected from the group consisting of: (a) a polynucleotide encoding the amino acid sequence of SEQ ID NO:1; (b) a polynucleotide encoding a fragment of SEQ ID NO:1 wherein a fragment comprises at least 15 contiguous amino acids; (c) a polynucleotide complementary to (a); and (d) a polynucleotide complementary to (b).
2. An isolated and purified polynucleotide which hybridizes under stringent conditions to the polynucleotide of claim 1 wherein stringent conditions are wash conditions of 15 mM NaCl, 1.5 mM trisodium citrate, at 68° C.
3. An isolated and purified polynucleotide of claim 1, comprising a polynucleotide sequence selected from the group consisting of: (a) a polynucleotide encoding the amino acid sequence of SEQ ID NO:1; and (b) a polynucleotide complementary to (a).
4. An expression vector comprising the polynucleotide of claim 1.
5. A host cell comprising the expression vector of claim 4.
6. A method for producing a polypeptide comprising a sequence of SEQ ID NO:1, the method comprising the steps of: (a) culturing the host cell of claim 5 under conditions suitable for the expression of the polypeptide; and (b) recovering the polypeptide from the host cell culture.
7. An isolated and purified polynucleotide comprising a polynucleotide sequence selected from the group consisting of: (a) SEQ ID NO:2; (b) a fragment of SEQ ID NO:2 wherein a fragment comprises at least 30 contiguous nucleic acids; (c) a polynucleotide complementary to (a); and (d) a polynucleotide complementary to (b).
8. An isolated and purified polynucleotide that hybridizes under stringent conditions to the polynucleotide of claim 7 wherein stringent conditions are wash conditions of 15 mM NaCl, 1.5 mM trisodium citrate, at 68° C.
9. An isolated and purified polynucleotide of claim 7, comprising a polynucleotide sequence selected from the group consisting of: (a) SEQ ID NO:2; and (b) a polynucleotide complementary to (a).

L16 ANSWER 7 OF 15 USPATFULL on STN

2000:74131 Recombinant **dengue** virus DNA fragment.

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The United States of America as represented by the Secretary of the Army,

Washington, DC, United States (U.S. government)

US 6074865 20000613

**APPLICATION: US 1995-504878 19950720 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB A recombinant protein encompassing the complete envelope glycoprotein and a portion of the carboxy-terminus of the membrane/premembrane protein of **dengue** 2 virus was expressed in baculovirus as a protein particle. The recombinant protein particle was purified and found to provide protection against lethal challenge with **dengue** 2 virus in mice.

CLM What is claimed is:

1. An isolated and purified **dengue** virus DNA fragment consisting essentially of a DNA fragment which encodes a complete **dengue** virus envelope protein and a carboxy terminus segment of premembrane protein which comprises a translocation signal for said Envelope protein.
2. The isolated and purified DNA fragment according to claim 1, wherein said **dengue** virus is **dengue** 2.
3. The DNA fragment of claim 2 which encodes 495 amino acids of said envelope protein and 31 amino acids of said carboxy terminus segment of premembrane protein, said fragment comprising the nucleotide sequence specified in SEQ ID NO: 1 or an allelic variant which retains the neutralizing antibody production characteristic of a protein encoded by SEQ ID No. 1.
4. The DNA fragment according to claim 3, wherein said DNA fragment encodes the amino acid sequence specified in SEQ ID NO: 2.
5. The isolated and purified DNA fragment according to claim 1, wherein said **dengue** is **dengue** 1.



said **dengue** is selected from the group consisting of **dengue** 3 and **dengue** 4.

7. A recombinant DNA construct comprising: (i) a vector, and (ii) an isolated and purified **dengue** virus DNA fragment according to claim 1.

8. A recombinant DNA construct according to claim 7, wherein said **dengue** virus is **dengue** 2.

9. The recombinant DNA construct according to claim 7, wherein said vector is a eukaryotic expression vector.

10. The recombinant DNA construct according to claim 8, wherein said vector is a eukaryotic expression vector.

11. A recombinant DNA construct comprising: (i) a vector, and (ii) a **dengue** 2 DNA fragment according to claim 3.

12. The recombinant DNA construct according to claim 11, wherein said vector is a eukaryotic expression vector.

13. The recombinant DNA construct according to claim 11, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO: 2.

14. The recombinant DNA construct according to claim 11 wherein said vector is pBlueBacIII.

15. A host cell transformed with a recombinant DNA construct comprising: (i) a vector, and (ii) an isolated and purified **dengue** virus DNA fragment according to claim 1.

16. A host cell according to claim 15, wherein said cell is prokaryotic.

17. The host cell according to claim 15, wherein said cell is a eukaryotic cell.

18. A method for producing a **dengue** virus recombinant protein particle, said method comprising the steps of: (i) culturing a host cell transformed with an expression vector according to claim 9 under conditions such that said DNA fragment is expressed and said recombinant protein is produced as a particle, said particle comprising more than one unit of said recombinant protein; and (ii) isolating said recombinant protein particle.

19. The method according to claim 18, wherein said **dengue** virus is **dengue** 2.

20. The method of claim 18 wherein isolating said recombinant protein particle comprises: (i) pelleting said cells by centrifugation, (ii) separating the cell pellet and the supernatant, (iii) lysing said cell pellet to release said recombinant protein particle; (iv) isolating said recombinant protein particle of step (iii); (v) fractionating said recombinant protein particle of step (iv) on a gradient; and (vi) isolating said recombinant protein particle, in a purified form.

L16 ANSWER 8 OF 15 USPTAFULL on STN

2000:9526 cDNA sequence of **Dengue** virus serotype 1 (Singapore strain).

Fu, Jianlin, Singapore, Singapore

Tan, Boon-Huan, Singapore, Singapore

Yap, Eu-Hian, Singapore, Singapore

Chan, Yow-Cheong, Singapore, Singapore

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Institute of Molecular and Cell Biology, Singapore (non-U.S. corporation)

US 6017535 20000125

WO 9322440 19931111

**APPLICATION: US 1994-325426 19941216 (8)**

WO 1993-CA182 19930428 19941216 PCT 371 date 19941216 PCT 102(e) date

PRIORITY: GB 1992-9243 19920429

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB DENI-S275/90 (ECACC V92042111) is a new strain of **Dengue** virus serotype 1. The complete cDNA sequence of this virus has been cloned and protein-coding fragments thereof have been used in the construction of expression plasmids. DENI-S275/90 in inactivated form, DENI-S275/90 polypeptides or fusion proteins thereof can be incorporated into vaccines for immunisation against DENI-S275/90 and other DENI viruses. The invention further provides diagnostic reagents e.g. labelled antibodies to DENI-S275/90 proteins, and kits to detect DENI virus.

CLM What is claimed is:

1. An isolated **Dengue** viral strain DENI-S275/90 designated as (E-CACC V92042111).

2. An isolated **Dengue** viral strain DEN1-S275/90 designated as (ECACC V92042111), in inactivated form.
3. An isolated DNA polynucleotide encoding DEN-1-S275/90 (ECACC V92042111) consisting of the sequence shown in SEQ ID NO:1 or at least one nucleotide sequence selected from the group consisting of nucleic acids 81-422, 123-422, 423-695, 696-920, 921-2402, 2403-3464, 3465-4112, 4113-4499, 4500-6359, 6360-6809, 6810-7556, and 7557-10268, of SEQ ID NO:1.
4. An isolated DNA polynucleotide encoding the polypeptide of SEQ ID NO:2 or at least one amino acid sequence selected from the group consisting of amino acids 1-114, 15-114, 115-205, 206-280, 281-774, 775-1128, 1129-1344, 1345-1474, 1475-2093, 2094-2242, 2243-2492 and 2493-3396 of SEQ ID NO:2.
5. An isolated DNA polynucleotide encoding a fusion protein comprising the polypeptide of SEQ ID NO: 2 or at least one amino acid sequence selected from the group consisting of amino acids 1-114, 15-114, 115-205, 206-280, 281-774, 775-1128, 1129-1344, 1345-1474, 1475-2093, 2094-2242, 2243-2492 and 2493-3396 of SEQ ID NO:2.
6. An expression vector comprising the DNA polynucleotide of any one of claims 3-4.
7. The expression vector according to claim 6, said vector being a plasmid or viral vector.
8. An expression vector comprising the isolated DNA polynucleotide of claim 5.
9. A cell containing the expression vector according to claim 6.
10. The cell according to claim 9, said cell being *E. coli*, a yeast cell or an insect cell.
11. A cell containing the expression vector of claim 8.
12. A method of preparing a polypeptide having an amino acid sequence selected from the group consisting of amino acids 1-114, 15-114, 115-205, 206-280, 281-774, 775-1128, 1129-1344, 1345-1474, 1475-2093, 2094-2242, 2243-2492 and 2493-3396, of SEQ ID NO:2, said method comprising culturing a cell according to claim 9 and recovering said polypeptide.
13. A method of preparing a fusion protein comprising the polypeptide of SEQ ID NO: 2 or at least one amino acid sequence selected from the group consisting of amino acids 1-114, 15-114, 115-205, 206-280, 281-774, 775-1128, 1129-1344, 1345-1474, 1475-2093, 2094-2242, 2243-2492 and 2493-3396 of SEQ ID NO:2, said method comprising culturing a cell according to claim 11 and recovering said fusion protein.
14. A polypeptide in isolated form which is selected from the group consisting of amino acids 1-14, 15-114, 115-205, 206-280, 281-774, 775-1128, 1129-1344, 345-1474, 1475-2093, 2094-2242, 2243-2492 and 2493-3396, of SEQ ID NO:2.
15. An isolated fusion protein comprising the polypeptide according to claim 14.
16. A polypeptide according to claim 14, attached to a label.
17. A fusion protein according to claim 15 attached to a label.
18. A test kit for the detection of the presence or absence of DEN1 virus antibodies comprising a polypeptide according to any one of claims 14-17 fixed to a solid support, said polypeptide being capable of binding antibodies to DEN1-S275/90 (ECACC V92042111).
19. A method of preparing antibodies in an animal, said antibodies being capable of binding a **Dengue** virus viral protein, said method comprising immunizing said animal with at least one polypeptide according to any one of claims 14-17 or the inactivated virus according to claim 2 and isolating said antibodies.
20. A method as claimed in claim 19 which further comprises in vitro labeling one or more isolated antibodies capable of binding a **Dengue** viral protein.
21. An isolated antibody prepared according to the method of claim 19.

1998:68530 Trova fowl pox virus recombinants comprising heterologous inserts.

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Tartaglia, James, Schenectady, NY, United States  
Norton, Elizabeth K., Latham, NY, United States  
Riviere, Michel, Ecully, France  
de Taisne, Charles, Lyons, France  
Limbach, Keith J., Troy, NY, United States  
Johnson, Gerard P., Waterford, NY, United States  
Pincus, Steven E., East Greenbush, NY, United States  
Cox, William I., Troy, NY, United States  
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Virogenetics Corporation, Troy, NY, United States (U.S. corporation)  
US 5766599 19980616

**APPLICATION: US 1995-458101 19950601 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB What is described is a modified vector, such as a recombinant poxvirus, particularly recombinant vaccinia virus, having enhanced safety. The modified recombinant virus has nonessential virus-encoded genetic functions inactivated therein so that virus has attenuated virulence. In one embodiment, the genetic functions are inactivated by deleting an open reading frame encoding a virulence factor. In another embodiment, the genetic functions are inactivated by insertional inactivation of an open reading frame encoding a virulence factor. What is also described is a vaccine containing the modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the vaccine has an increased level of safety compared to known recombinant virus vaccines.

CLM What is claimed is:

1. An attenuated virus having all the identifying characteristics of: a TROVAC fowlpox virus.
2. A virus which is TROVAC.
3. A vector which comprises the virus of claim 1.
4. A vector which comprises the virus of claim 2.
5. A virus as claimed in claim 2 further comprising exogenous DNA from a non-poxvirus source in a nonessential region of the virus genome.
6. A virus as claimed in claim 5 wherein the exogenous DNA is selected from the group consisting of rabies virus, Hepatitis B virus, **Japanese encephalitis virus, yellow fever virus, Dengue virus**, measles virus, pseudorabies virus, Epstein-Barr virus, herpes simplex virus, human immunodeficiency virus, simian immunodeficiency virus, equine herpes virus, bovine herpes virus, bovine viral diarrhea virus, human cytomegalovirus, canine parvovirus, equine influenza virus, feline leukemia virus, feline herpes virus, Hantaan virus, C. tetani, avian influenza virus, mumps virus and Newcastle Disease virus.
7. A virus as claimed in claim 6 wherein the non-poxvirus source is avian influenza virus and the fowlpox virus is vFP89, vFP92, vFP100 or vFP122.
8. A virus as claimed in claim 6 wherein the virus is a fowlpox virus, the non-poxvirus source is human immunodeficiency virus and the fowlpox virus is vFP62, vFP63 or vFP174.
9. A virus as claimed in claim 6 wherein the non-poxvirus source is Newcastle Disease virus and the fowlpox virus is vFP96.
10. A virus as claimed in claim 6 which is a human immunodeficiency virus recombinant fowlpox virus which is vFP62 or vFP63.
11. A virus as claimed in claim 1 further comprising exogenous DNA from a non-poxvirus source in a nonessential region of the virus genome.
12. An immunological composition for inducing an immunological response in a host animal inoculated with said composition, said composition comprising the virus of any one of claims 1, 2 or 10 or 11, or, a vector as claimed in claim 3 or 4, and a carrier.
13. The immunological composition of claim 12 containing the virus or vector in an amount sufficient to induce a protective immunological response such that the immunological composition is a vaccine.
14. A method of expressing a gene product in a cell cultured in vitro comprising introducing into the cell a virus as claimed in any one of

transforming cell with the expression vector, cultivating the transformed cell under conditions which allow expression of the gene product, and further purifying the product.

L16 ANSWER 10 OF 15 USPATFULL on STN

1998:64734 Modified recombinant vaccinia virus and expression vectors thereof.

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Taylor, Jill, Albany, NY, United States  
Tartaglia, James, Schenectady, NY, United States  
Norton, Elizabeth K., Latham, NY, United States  
Riviere, Michel, Ecully, France  
de Taisne, Charles, Lyon, France  
Limbach, Keith J., Troy, NY, United States  
Johnson, Gerard P., Waterford, NY, United States  
Pincus, Steven E., East Greenbush, NY, United States  
Cox, William I., Troy, NY, United States  
Audonnet, Jean-Christophe Francis, Albany, NY, United States  
Gettig, Russell Robert, Averill Park, NY, United States  
Virogenetics Corporation, Troy, NY, United States (U.S. corporation)  
US 5762938 19980609

**APPLICATION: US 1996-709209 19960821 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB What is described is a modified vector, such as a recombinant poxvirus, particularly recombinant vaccinia virus, having enhanced safety. The modified recombinant virus has nonessential virus-encoded genetic functions inactivated therein so that virus has attenuated virulence. In one embodiment, the genetic functions are inactivated by deleting an open reading frame encoding a virulence factor. In another embodiment, the genetic functions are inactivated by insertional inactivation of an open reading frame encoding a virulence factor. What is also described is a vaccine containing the modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the vaccine has an increased level of safety compared to known recombinant virus vaccines.

CLM What is claimed is:

1. A recombinant vaccinia virus wherein regions C7L-K3L, J2R, B13R+B14R, A26L, A56R and I4L have been deleted therefrom.

2. A recombinant vaccinia virus wherein regions C23L-F4L, J2R, B13R+B14R, A26L, A56R and I4L have been deleted therefrom.

3. A recombinant vaccinia virus wherein regions C7L-K1L, J2R, B13R-B29R, A26L, A56R and I4L have been deleted therefrom.

4. A recombinant vaccinia wherein regions C23L-F4L, J2R, B13R-B29R, A26L, A56 and I4L have been deleted therefrom.

5. The recombinant vaccinia virus of claim 1 including exogenous DNA from a non-vaccinia source.

6. The recombinant vaccinia virus of claim 2 including exogenous DNA from a non-vaccinia source.

7. The recombinant vaccinia virus of claim 3 including exogenous DNA from a non-vaccinia source.

8. The recombinant vaccinia virus of claim 4 including exogenous DNA from a non-vaccinia source.

9. A recombinant vaccinia virus selected from the group consisting of: vP954, vP938, vP953, vP977, vP996, vP1006 and vP1015.

10. A method for expressing a gene product in a cell cultured in vitro, which method comprises introducing into the cell the recombinant vaccinia virus as claimed in any of claims 1, 2, 3, 4, 5, 6, 7, 8 or 9, and culturing the cell under appropriate conditions for expression of the gene product.

11. An immunological composition comprising a carrier and a recombinant vaccinia virus as claimed in any of claims, 1, 2, 3, 4, 5, 6, 7, 8, 8 or 9.

L16 ANSWER 11 OF 15 USPATFULL on STN

1998:57530 Alvac canarypox virus recombinants comprising heterologous inserts.

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Perkus, Marion E., Altamont, NY, United States  
Taylor, Jill, Albany, NY, United States  
Tartaglia, James, Schenectady, NY, United States

Riviere, Michel, Ecully, France  
de Taisne, Charles, Lyons, France  
Limbach, Keith J., Troy, NY, United States  
Johnson, Gerard P., Waterford, NY, United States  
Pincus, Steven E., East Greenbush, NY, United States  
Cox, William I., Troy, NY, United States  
Audonnet, Jean-Christophe Francis, Albany, NY, United States  
Gettig, Russell Robert, Averill Park, NY, United States  
Virogenetics Corporation, Troy, NY, United States (U.S. corporation)  
US 5756103 19980526

APPLICATION: US 1995-457007 19950601 (8)

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB What is described is a modified vector, such as a recombinant poxvirus, particularly recombinant vaccinia virus, having enhanced safety. The modified recombinant virus has nonessential virus-encoded genetic functions inactivated therein so that virus has attenuated virulence. In one embodiment, the genetic functions are inactivated by deleting an open reading frame encoding a virulence factor. In another embodiment, the genetic functions are inactivated by insertional inactivation of an open reading frame encoding a virulence factor. What is also described is a vaccine containing the modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the vaccine has an increased level of safety compared to known recombinant virus vaccines.

CLM What is claimed is:

1. An attenuated virus having all the identifying characteristics of: an ALVAC canarypox virus.
2. A virus which is ALVAC.
3. A vector which comprises the virus of claim 1.
4. A vector which comprises the virus of claim 2.
5. A virus as claimed in claim 2 further comprising exogenous DNA from a non-poxvirus source in a nonessential region of the virus genome.
6. A virus as claimed in claim 5 wherein the exogenous DNA is selected from the group consisting of rabies virus, Hepatitis B virus, **Japanese encephalitis virus, yellow fever virus, Dengue virus**, measles virus, pseudorabies virus, Epstein-Barr virus, herpes simplex virus, human immunodeficiency virus, simian immunodeficiency virus, equine herpes virus, bovine herpes virus, bovine viral diarrhea virus, human cytomegalovirus, canine parvovirus, equine influenza virus, feline leukemia virus, feline herpes virus, Hantaan virus, C. tetani, avian influenza virus, mumps virus and Newcastle Disease virus.
7. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is rabies virus, and the canarypox virus is vCP65 or vCP136.
8. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is human immunodeficiency virus and the canarypox virus is vCP95, vCP112, vCP60, vCP61, vCP125, vCP124, vCP126, vCP144, vCP120, vCP138, vCP117, vCP130, vCP152, vCP155, vCP156, vCP146, vCP148, vCP154, vCP168 or vCP153.
9. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is equine herpes virus and the canarypox virus is vCP132.
10. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is human cytomegalovirus and the canarypox virus is vCP139.
11. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is canine parvovirus and the canarypox virus is vCP123 or vCP136.
12. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is Epstein-Barr virus and the canarypox virus is vCP167.
13. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is equine influenza virus and the canarypox virus is vCP128 or vCP159.
14. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is feline leukemia virus and the canarypox virus is vCP177, vCP83, vCP35, vCP37, vCP87, vCP93 or vCP97.

virus, the non-poxvirus source is feline herpes virus and the canarypox virus is vCP162.

16. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is Hantaan virus and the canarypox virus is vCP114 or vCP119.

17. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is Hepatitis B and the canarypox virus is vCP169 or vCP157.

18. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is C. tetani and the canarypox virus is vCP161.

19. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is mumps virus and the canarypox virus is vCP171.

20. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is **Japanese encephalitis** virus and the canarypox virus is vCP107 or vCP140.

21. A virus as claimed in claim 6 wherein the virus is a canarypox virus, the non-poxvirus source is simian immunodeficiency virus, and the canarypox virus is vCP172.

22. A virus as claimed in claim 6 which is a rabies virus recombinant canarypox virus which is vCP65.

23. A virus as claimed in claim 6 which is a human immunodeficiency virus recombinant canarypox virus which is vCP95, vCP112, vCP60 or vCP61.

24. A virus as claimed in claim 1 further comprising exogenous DNA from a non-poxvirus source in a non-essential region of the virus genome.

25. An immunological composition for inducing an immunological response in a host animal inoculated with said composition, said composition comprising the virus of any one of claims 1, 2 or 5 to 24, or, a vector as claimed in claim 3 or 4, and a carrier.

26. The immunological composition of claim 25 which is a vaccine.

27. A method of expressing a gene product in a cell cultured in vitro comprising introducing into the cell a virus as claimed in any one of claims 1, 2 or 5 to 24, or, a vector, transforming cell with the expression vector, cultivating the transformed cell under conditions which allow expression of the recombinant poxvirus, and further purifying the protein as claimed in claim 3 or 4.

L16 ANSWER 12 OF 15 USPTAFULL on STN

1998:44886 **Flavivirus** recombinant poxvirus immunological composition.

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Pincus, Steven Elliot, East Greenbush, NY, United States

Virogenetics Corporation, Troy, NY, United States (U.S. corporation)

US 5744141 19980428

**APPLICATION: US 1995-484304 19950607 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB What is described is a recombinant poxvirus, such as vaccinia virus, fowlpox virus and canarypox virus, containing foreign DNA from **flavivirus**, such as **Japanese encephalitis** virus, **yellow fever virus** and **Dengue** virus. In a preferred embodiment, the recombinant poxvirus generates an extracellular particle containing **flavivirus E** and M proteins capable of inducing neutralizing antibodies, hemagglutination-inhibiting antibodies and protective immunity against **flavivirus** infection. What is also described is a vaccine containing the recombinant poxvirus for inducing an immunological response in a host animal inoculated with the vaccine.

CLM What is claimed is:

1. A recombinant poxvirus comprising DNA coding for at least one **flavivirus** structural protein, wherein the **flavivirus** is **Yellow Fever virus** or **Dengue** virus and the poxvirus is selected from the group consisting of: an avipox virus, a vaccinia virus wherein the open reading frames for the thymidine kinase gene, a hemorrhagic region, an A type inclusion body region, a hemagglutinin gene, a host range gene region and a large subunit, ribonucleotide reductase have been deleted therefrom, a vaccinia virus wherein regions C7L-K1L, J2R, B13R+B14R, A26L, A56R, and I4L have been deleted therefrom, and a NYVAC vaccinia virus.

2. The recombinant poxvirus of claim 1 wherein the DNA comprises a part of the **flavivirus** open reading frame from c to NS2b.
3. The recombinant poxvirus of claim 1 wherein the DNA encodes protein M or a precursor to protein M, and **flavivirus** proteins **E**, NS1 and NS2A.
4. The recombinant poxvirus of claim 1 wherein the poxvirus is a vaccinia virus.
5. The recombinant poxvirus of claim 1 wherein the poxvirus is an avipox virus.
6. The recombinant poxvirus of claim 5 wherein the avipox virus is canarypox virus.
7. The recombinant poxvirus of claim 1 wherein the **flavivirus** is **Yellow Fever virus**.
8. The recombinant poxvirus of claim 1 wherein the **flavivirus** is **Dengue** virus.
9. The recombinant poxvirus of claim 6 wherein the canarypox virus is an ALVAC canarypox virus.
10. The recombinant poxvirus of claim 6 wherein the canarypox virus is attenuated through more than 200 serial passages on chick embryo fibroblasts, a master seed therefrom was subjected to four successive plaque purifications under agar, from which a plaque clone was amplified through five additional passages.
11. The recombinant poxvirus of claim 4 wherein in the vaccinia virus, the open reading frames for the thymidine kinase gene, a hemorrhagic region, an A type inclusion body region, a hemagglutinin gene, a host range gene region and a large subunit, ribonucleotide reductase have been deleted therefrom, or regions C7L-K1L, J2R, B13R+B14R, A26L, A56R, and I4L have been deleted therefrom.
12. The recombinant poxvirus of claim 4 wherein the vaccinia virus is a NYVAC vaccinia virus.
13. The recombinant poxvirus of claim 1 which is vCP127 or vCP107.
14. The recombinant poxvirus of claim 1 wherein the DNA comprises DNA encoding C-terminal amino acids of C.
15. The recombinant poxvirus of claim 1 wherein the DNA further comprises DNA encoding NS2b.
16. An immunological composition comprising a carrier and a recombinant poxvirus according to any one of claims 1-15, wherein the composition is effective to induce an immunological response in a host.
17. A method for producing a **flavivirus** structural protein comprising introducing into a cell a recombinant poxvirus, transforming cell with the expression vector, cultivating the transformed cell under conditions which allow expression of the recombinant poxvirus, and further purifying the protein as claim in any one of claims 1-15.

L16 ANSWER 13 OF 15 USPATFULL on STN

1998:44885 **Flavivirus** recombinant poxvirus vaccine.

Paoletti, Enzo, Delmar, NY, United States

Pincus, Steven Elliot, East Greenbush, NY, United States

Virgenetics Corporation, Troy, NY, United States (U.S. corporation)

US 5744140 19980428

**APPLICATION: US 1994-224391 19940407 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB What is described is a recombinant poxvirus, such as vaccinia virus, fowlpox virus and canarypox virus, containing foreign DNA from **flavivirus**, such as **Japanese encephalitis** virus, **yellow fever virus** and **Dengue** virus. In a preferred embodiment, the recombinant poxvirus generates an extracellular particle containing **flavivirus E** and M proteins capable of inducing neutralizing antibodies, hemagglutination-inhibiting antibodies and protective immunity against **flavivirus** infection. What is also described is a vaccine containing the recombinant poxvirus for inducing an immunological response in a host animal inoculated with the vaccine.

CLM What is claimed is:

1. A recombinant avipox virus comprising DNA coding for **Japanese encephalitis** virus protein M or a precursor to protein M, and **Japanese encephalitis** virus protein **E**, NS1 and NS2A, in a

2. A recombinant avipox virus as in claim 1 wherein the avipox virus is canarypox virus.
3. A recombinant avipox virus as in claim 2 wherein the canarypox virus is ALVAC or a canarypox virus attenuated through more than 200 serial passages on chick embryo fibroblasts, subjected to four successive plaque purifications, and amplified through five additional serial passages.
4. A recombinant avipox virus comprising DNA from **Japanese encephalitis** virus (JEV) in a nonessential region of the avipox genome, wherein the DNA comprises the part of the JEV open reading frame extending from **prM** to NS2a.
5. A recombinant avipox virus as in claim 4, wherein the part of the JEV open reading frame further comprises the DNA encoding 15 C-terminal amino acids of C.
6. A recombinant avipox virus as in claim 4, wherein the part of the JEV open reading frame further comprises NS2b.
7. The recombinant avipox virus of claim 4 which is a canarypox virus which is ALVAC or a canarypox virus attenuated through more than 200 serial passages on chick embryo fibroblasts, subjected to four successive plaque purifications, and amplified through five additional serial passages.
8. An immunological composition comprising a carrier and an avipox virus according to claim 1, wherein the composition is effective to induce an immunological response in a host animal.
9. An immunological composition comprising a carrier and an avipox virus according to claim 3, wherein the composition is effective to induce an immunological response in a host animal.
10. An immunological composition comprising a carrier and an avipox virus according to claim 4, wherein the composition is effective to induce an immunological response in a host animal.
11. An immunological composition comprising a carrier and an avipox virus according to claim 7, wherein the composition is effective to induce an immunological response in a host animal.
12. A vaccine composition comprising a carrier and an avipox virus according to claim 1, wherein the composition is effective to induce a protective response against **Japanese encephalitis** virus in a host animal.
13. A vaccine composition comprising a carrier and an avipox virus according to claim 3, wherein the composition is effective to induce a protective response against **Japanese encephalitis** virus in a host animal.
14. A vaccine composition comprising a carrier and an avipox virus according to claim 4, wherein the composition is effective to induce a protective response against **Japanese encephalitis** virus in a host animal.
15. A vaccine composition comprising a carrier and an avipox virus according to claim 7, wherein the composition is effective to induce a protective response against **Japanese encephalitis** virus in a host animal.
16. The recombinant avipox virus of claim 4 which is vcP107.

L16 ANSWER 14 OF 15 USPTAFULL on STN

96:38606 **Flavivirus** recombinant poxvirus vaccine.

Paoletti, Enzo, Delmar, NY, United States

Pincus, Steven E., East Greenbush, NY, United States

Virogenetics Corporation, Troy, NY, United States (U.S. corporation)

US 5514375 19960507

**APPLICATION: US 1991-714687 19910613 (7)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB What is described is a recombinant poxvirus, such as vaccinia virus, fowlpox virus and canarypox virus, containing foreign DNA from **flavivirus**, such as **Japanese encephalitis** virus, **yellow fever virus** and **Dengue** virus. In a preferred embodiment, the recombinant poxvirus generates an extracellular particle containing **flavivirus E** and M proteins capable of inducing neutralizing antibodies,



**flavivirus** infection. What is also described is a vaccine containing the recombinant poxvirus for inducing an immunological response in a host animal inoculated with the vaccine.

What is claimed is:

1. A recombinant vaccinia virus comprising DNA coding for **Japanese encephalitis** virus protein M or a precursor to protein M, and **Japanese encephalitis** virus proteins E, NS1 and NS2A, in a nonessential region of the vaccinia genome.
2. The recombinant vaccinia virus of claim 1 wherein regions C7L-K1L, J2R, B13R+B14R, A26L, A56R, and I4L have been deleted therefrom.
3. The recombinant vaccinia virus of claim 1 wherein the open reading frames for the thymidine kinase gene, the hemorrhagic region, the A type inclusion body region, the hemagglutinin gene, the host range gene region, and the large subunit, ribonucleotide reductase have been deleted therefrom.
4. An immunological composition which induces an immunological response in a host animal inoculated with said composition comprising a carrier and a recombinant vaccinia virus as claimed in claim 1.
5. An immunological composition which induces an immunological response in a host animal inoculated with said composition comprising a carrier and a recombinant vaccinia virus as claimed in claim 2.
6. A recombinant vaccinia virus as in claim 2 wherein the poxvirus is a NYVAC recombinant vaccinia virus.
7. An immunological composition which induces an immunological response in a host animal inoculated with said composition comprising a carrier and a recombinant vaccinia virus as claimed in claim 6.
8. An immunological composition which induces an immunological response in a host animal inoculated with said composition comprising a carrier and a recombinant vaccinia virus as claimed in claim 3.
9. A recombinant vaccinia virus as in claim 3 wherein the vaccinia virus is a NYVAC recombinant vaccinia virus.
10. A vaccine which induces an immunological response in a host animal inoculated with said vaccine, said vaccine comprising a carrier and a recombinant vaccinia virus as claimed in claim 7.
11. A recombinant vaccinia virus as in claim 1 which is selected from the group consisting of vP650, vP555, and vP908.
12. A vaccine which induces an immunological response in a host animal inoculated with said vaccine, said vaccine comprising a carrier and a recombinant vaccinia virus as claimed in claim 1.
13. A vaccine which induces an immunological response in a host animal inoculated with said vaccine, said vaccine comprising a carrier and a recombinant vaccinia virus as claimed in claim 2.
14. A vaccine for which induces an immunological response in a host animal inoculated with said vaccine, said vaccine comprising a carrier and a recombinant vaccinia virus as claimed in claim 3.
15. A recombinant vaccinia virus wherein regions C7L-K1L, J2R, B13R+B14R, A26L, A56R, and I4L have been deleted therefrom, and further comprising DNA from **Japanese encephalitis** virus in a non-essential region of the vaccinia genome.
16. A recombinant vaccinia virus as in claim 15 wherein the vaccinia virus is a NYVAC recombinant vaccinia virus.
17. A recombinant vaccinia virus wherein the open reading frames for the thymidine kinase gene, the hemorrhagic region, the A type inclusion body region, the hemagglutinin gene, the host range gene region, and the large subunit, ribonucleotide reductase have been deleted therefrom, and further comprising DNA from **Japanese encephalitis** virus in a non-essential region of the vaccinia genome.
18. A recombinant vaccinia virus as in claim 17 wherein the vaccinia virus is a NYVAC recombinant vaccinia virus.
19. A recombinant vaccinia virus as claimed in claim 17 which is: vP923.
20. A recombinant vaccinia virus comprising DNA from **Japanese encephalitis** virus in a nonessential region of the vaccinia genome wherein the DNA codes for a precursor to **Japanese encephalitis** virus

NS2A; or, the DNA codes for **Japanese encephalitis** virus proteins NS1 and NS2A; or, the DNA codes for **Japanese encephalitis** virus proteins NS1, NS2A and NS2B.

21. A recombinant vaccinia virus as claimed in claim 20 which is: vP825, vP857 or vP864.

22. A recombinant vaccinia virus as in claim 6 which is vP908.

L16 ANSWER 15 OF 15 USPTAFULL on STN

96:16887 NYVAC vaccinia virus recombinants comprising heterologous inserts.

Paoletti, Enzo, Delmar, NY, United States  
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Norton, Elizabeth K., Latham, NY, United States  
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de Taisne, Charles, Lyons, France  
Limbach, Keith J., Troy, NY, United States  
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Gettig, Russell R., Averill Park, NY, United States  
Virogenetics Corporation, Troy, NY, United States (U.S. corporation)  
US 5494807 19960227

**APPLICATION: US 1993-105483 19930812 (8)**

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB What is described is a modified vector, such as a recombinant poxvirus, particularly recombinant vaccinia virus, having enhanced safety. The modified recombinant virus has nonessential virus-encoded genetic functions inactivated therein so that virus has attenuated virulence. In one embodiment, the genetic functions are inactivated by deleting an open reading frame encoding a virulence factor. In another embodiment, the genetic functions are inactivated by insertional inactivation of an open reading frame encoding a virulence factor. What is also described is a vaccine containing the modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the vaccine has an increased level of safety compared to known recombinant virus vaccines.

CLM What is claimed is:

1. A recombinant vaccinia virus wherein regions C7L-K1L, J2R, B13R+B14R, A26L, A56R and I4L have been deleted therefrom, and further comprising exogenous coding DNA from a non-vaccinia source in a nonessential region of the vaccinia genome.

2. A recombinant vaccinia virus wherein the open reading frames for the thymidine kinase gene, the hemorrhagic region, the A type inclusion body region, the hemagglutinin gene, the host range gene region, and, the large subunit, ribonucleotide reductase have been deleted therefrom, and further comprising exogenous coding DNA from a non-vaccinia source in a nonessential region of the vaccinia genome.

3. A recombinant vaccinia virus as claimed in claim 2 wherein the non-vaccinia source is selected from the group consisting of rabies virus, Hepatitis B virus, **yellow fever virus**, Dengue virus, pseudorabies virus, Epstein-Barr virus, herpes simplex virus, simian immunodeficiency virus, equine herpes virus, bovine herpes virus, bovine viral diarrhea virus, human cytomegalovirus, canine parvovirus, equine influenza virus, feline leukemia virus, feline herpes virus, Hantaan virus, C. tetani, avian influenza virus, mumps virus and Newcastle Disease virus.

4. A recombinant vaccinia virus as claimed in claim 3, wherein the non-vaccinia source is rabies virus and the recombinant vaccinia virus is vP879 or vP999.

5. A recombinant vaccinia virus as claimed in claim 3, wherein the non-vaccinia source is Hepatitis B virus and the recombinant vaccinia virus is vP856, vP896, vP897, vP858, vP891, vP932, vP975, vP930, vP919, vP941 or vP944.

6. A recombinant vaccinia virus as claimed in claim 3, wherein the non-vaccinia source is **yellow fever virus** and the recombinant vaccinia virus is vP766, vP764, vP869, vP729, vP725, vP997, or vP984.

7. A recombinant vaccinia virus as claimed in claim 3, wherein the non-vaccinia source is **Dengue** virus and the recombinant vaccinia virus is vP867, vP962 or vP955.

non-vaccinia source is pseudorabies virus and the recombinant vaccinia virus is vP881, vP883, vP900, vP912, vP925, vP915 or vP916.

9. A recombinant vaccinia virus as claimed in claim 3, wherein the non-vaccinia source is Epstein-Barr virus and the recombinant vaccinia virus is vP941 or vP944.

10. A recombinant vaccinia virus as claimed in claim 3, wherein the non-vaccinia source is herpes simplex virus and the recombinant vaccinia virus is vP914.

11. A recombinant vaccinia virus as claimed in claim 3, wherein the non-vaccinia source is simian immunodeficiency virus and the recombinant vaccinia virus is vP873, vP948, vP943, vP942, vP952, vP948, vP1042, vP1071, vP943, vP942, vP952 or vP1050.

12. A recombinant vaccinia virus as claimed in claim 3 wherein the non-vaccinia source is equine herpes virus and the recombinant vaccinia virus is vP1043, vP1025 or vP956.

13. A recombinant vaccinia virus as claimed in claim 3 wherein the non-vaccinia source is bovine herpes virus and the recombinant vaccinia virus is vP1051, vP1074, vP1073, vP1083, vP1087 or vP1079.

14. A recombinant vaccinia virus as claimed in claim 3 wherein the non-vaccinia source is bovine viral diarrhea virus and the recombinant vaccinia virus is vP972, vP1017 or vP1097.

15. A recombinant vaccinia virus as claimed in claim 3 wherein the non-vaccinia source is human cytomegalovirus and the recombinant vaccinia virus is vP1001.

16. A recombinant vaccinia virus as claimed in claim 3 wherein the non-vaccinia source is canine parvovirus and the recombinant vaccinia virus is vP998 or vP999.

17. A recombinant vaccinia virus as claimed in claim 3 wherein the non-vaccinia source is equine influenza virus and the recombinant vaccinia virus is vP961 or vP1063.

18. A recombinant vaccinia virus as claimed in claim 3 wherein the non-vaccinia source is feline leukemia virus and the recombinant vaccinia virus is vP1011.

19. A recombinant vaccinia virus as claimed in claim 3 wherein the non-vaccinia source is Hantaan virus and the recombinant vaccinia virus is vP882, vP950 or vP951.

20. A recombinant vaccinia virus as claimed in claim 3 wherein the non-vaccinia source is C. tetani and the recombinant vaccinia virus is vP1075.

21. An immunological composition for inducing an immunological response in a host inoculated with the composition, said composition comprising a carrier and a recombinant virus as claimed in any one of claims 2, 33, 44, 5, 6, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20.

22. A method for expressing a gene product in a cell cultured in vitro, which method comprises introducing into the cell a modified recombinant virus as claimed in claim 2.

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FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

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L2      24958 S CMV OR CMV-IE
L3      3918 S L2 AND KOZAK
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<http://scientific.thomson.com/media/scpdf/ipcrdwpf.pdf> <<<

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E12	431	CHANG G S/IN

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L17 121 "CHANG G J"/IN

=> s l17 and (flavivir? or dengue or japanese encephalitis virus or yellow fever virus)

482 FLAVIVIR?  
 493 DENGUE  
 13912 JAPANESE  
 2191 ENCEPHALITIS  
 43600 VIRUS  
 168 JAPANESE ENCEPHALITIS VIRUS  
 (JAPANESE(W)ENCEPHALITIS(W)VIRUS)  
 37573 YELLOW  
 5880 FEVER  
 43600 VIRUS  
 190 YELLOW FEVER VIRUS  
 (YELLOW(W)FEVER(W)VIRUS)

L18 3 L17 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR  
 YELLOW FEVER VIRUS)

=> d l18, bib,ab,1-3

L18 ANSWER 1 OF 3 WPIDS COPYRIGHT 2006 THE THOMSON CORP on STN  
 Full Text  
 AN 2006-204378 [21] WPIDS

TI New isolated mutant **flavivirus** polypeptide, which exhibits measurably reduced antibody cross-reactivity, for treating or preventing **flavivirus** infection, e.g. **dengue** virus infection.

DC B04 D16 J04 S03

IN **CHANG, G J**; CRILL, W D

PA (USSH) US DEPT HEALTH & HUMAN SERVICES

CYC 111

PI WO 2006025990 A2 20060309 (200621)\* EN 151

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KM KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NA NG NI  
NO NZ OM PG PH PL PT RO RU SC SD SE SG SK SL SM SY TJ TM TN TR TT  
TZ UA UG US UZ VC VN YU ZA ZM ZW

ADT WO 2006025990 A2 WO 2005-US26672 20050727

PRAI US 2004-591898P 20040727

AB WO2006025990 A UPAB: 20060328

NOVELTY - An isolated mutant **flavivirus** polypeptide comprising any of 3 fully defined 495-501 amino acid sequences (SEQ ID NO: 14, 81, or 85), given in the specification, where at least one of the amino acids at position 104, 106, 107, 126, 226, or 231 is substituted compared to a wild-type **flavivirus** polypeptide, and where the polypeptide exhibits measurably reduced antibody cross-reactivity, is new.

DETAILED DESCRIPTION - INDEPENDENT CLAIMS are also included for:

- (1) an isolated nucleic acid molecule encoding the polypeptide;
- (2) a recombinant nucleic acid molecule comprising a regulatory sequence operably linked to the nucleic acid molecule;
- (3) a cell comprising the recombinant nucleic acid molecule;
- (4) a virus-like particle comprising the new polypeptide;
- (5) a method for identifying a **flavivirus** cross-reactive epitope;
- (6) a composition comprising the polypeptide or the nucleic acid vector, where the vector comprises the nucleic acid molecule, and carrier;
- (7) a method of eliciting an immune response against a **flavivirus** antigenic epitope in a subject;
- (8) a method of detecting a **flavivirus** antibody in a sample;
- (9) a method of diagnosing a **flavivirus** infection in a subject;
- (10) a **flavivirus** E-glycoprotein engineered to comprise at least one of the amino acid residue substitutions; and
- (11) a kit for detecting a **flavivirus** in a sample comprising the new polypeptide.

ACTIVITY - Virucide. No biological data given.

MECHANISM OF ACTION - Gene therapy.

USE - The mutant **flavivirus** polypeptides and nucleic acids are useful for eliciting an immune response against a **flavivirus** and for detecting **flaviviral** infection in humans. They can also be used for inhibition or treatment of **flaviviral** infection, e.g. **dengue** virus infection, **yellow fever virus** infection, **Japanese encephalitis virus** infection, St. Louis encephalitis virus infection, or West Nile virus infection (claimed).

Dwg.0/3

L18 ANSWER 2 OF 3 WPIDS COPYRIGHT 2006 THE THOMSON CORP on STN

Full Text

AN 2003-058572 [05] WPIDS

CR 2000-072885 [06]

DNN N2003-045382 DNC C2003-015067

TI Novel isolated nucleic acid useful as vaccine for preventing **flavivirus** infection, comprises transcriptional unit encoding signal sequence of one **flavivirus** and immunogenic **flavivirus** antigen of a second **flavivirus**.

DC B04 D16 S03

IN **CHANG, G J**; CHANG, G

PA (USSH-N) US DEPT HEALTH & HUMAN SERVICES; (USSH) US DEPT HEALTH & HUMAN SERVICES; (CHAN-I) CHANG G J

CYC 101

PI WO 2002081754 A1 20021017 (200305)\* EN 174

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RO RU SD SE SG SI SK SL TJ TM TN TR TT TZ UA UG US UZ VN YU ZA ZW

EP 1383931 A1 20040128 (200409) EN

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BR 2002008301 A 20040309 (200420)

KR 2003092051 A 20031203 (200424)

AU 2002307147 A1 20021021 (200433)

CN 1500152 A 20040526 (200458)

JP 2004532023 W 20041021 (200469) 237

NZ 529106 A 20050324 (200523)

US 2005163804 A1 20050728 (200550)  
 MX 2003008838 A1 20041201 (200561)  
 ADT WO 2002081754 A1 WO 2002-US10764 20020404; EP 1383931 A1 EP 2002-763960  
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 WO 2002-US10764 20020404; KR 2003092051 A KR 2003-713021 20031002; AU  
 2002307147 A1 AU 2002-307147 20020404; CN 1500152 A CN 2002-807758  
 20020404; JP 2004532023 W JP 2002-579516 20020404, WO 2002-US10764  
 20020404; NZ 529106 A NZ 2002-529106 20020404, WO 2002-US10764 20020404;  
 ZA 2003007580 A ZA 2003-7580 20030929; US 2005163804 A1 Cont of US  
 2001-826115 20010404, WO 2002-US10764 20020404, US 2004-500796 20040706;  
 MX 2003008838 A1 WO 2002-US10764 20020404, MX 2003-8838 20030929  
 FDT EP 1383931 A1 Based on WO 2002081754; BR 2002008301 A Based on WO  
 2002081754; AU 2002307147 A1 Based on WO 2002081754; JP 2004532023 W Based  
 on WO 2002081754; NZ 529106 A Based on WO 2002081754; MX 2003008838 A1  
 Based on WO 2002081754  
 PRAI US 2001-826115 20010404; US 2004-500796 20040706  
 AB WO 200281754 A UPAB: 20050923

NOVELTY - An isolated nucleic acid (I) comprising a transcriptional unit  
 encoding a signal sequence of a structural protein of a first **flavivirus**  
 and an immunogenic **flavivirus** antigen of a second **flavivirus**, where  
 the transcriptional unit directs the synthesis of the antigen, is new.

DETAILED DESCRIPTION - INDEPENDENT CLAIMS are also included for:

(1) a cell (II) comprising (I);

(2) a composition (III) comprising (I) and a pharmaceutically  
 acceptable carrier;

(3) an antigen (IV) produced from (I); and

(4) an antibody (V) produced in response to immunization by (IV).

ACTIVITY - Virucide.

MECHANISM OF ACTION - Vaccine (claimed).

Three-day (mixed sex) or 3-week-old (female) ICR outbred mice, 10 per  
 group, were vaccinated intramuscularly with 50 or 100 µg of nucleic acid  
 transcriptional unit (TU)-containing vaccine constructs, or subcutaneously  
 with doses of JE-VAX that were one-tenth or one-fifth the dose given to  
 humans. 3-day old vaccinated groups were challenged 7 weeks after  
 vaccination by intraperitoneal injection of 50000 plaque forming units  
 (pfu)/100 µl of the mouse-adapted **Japanese encephalitis virus**  
 (JEV) strain SA14 and observed for 3 weeks. 100% protection was achieved  
 in groups that received various nucleic acid TU-containing vaccine  
 constructs for up to 21 days. In contrast, 60% of the JE-VAX-vaccinated  
 mice did not survive virus challenge by 21 days. These results indicated  
 that the nucleic acid TU's of conferred effective protection on vaccinated  
 mice. This suggested the possibility of employing the nucleic acid vaccine  
 as an early childhood vaccine for humans. In contrast, JE-VAX, the  
 inactivated human vaccine currently used, did not appear to be effective  
 in young animals.

USE - (III) is useful for immunizing a subject against infection by a  
**flavivirus**, by administering an effective amount of (III) to the  
 subject. (IV) is useful for detecting **flavivirus** antibody in a sample,  
 by contacting the sample with (IV) under conditions to form an  
 antigen/antibody complex, and detecting antigen/antibody complex  
 formation, thus detecting a **flavivirus** antibody in the sample. (V) is  
 useful for detecting a **flavivirus** antigen in a sample, by contacting the  
 sample with (V) under conditions to form an antigen/antibody complex, and  
 detecting antigen/antibody complex formation, thus detecting a  
**flavivirus** antigen in the sample. (IV) or (V) is useful for diagnosing a  
**flavivirus** infection in a subject, by contacting the sample from the  
 subject with (IV) or (V) under conditions to form an antigen/antibody  
 complex, and detecting antigen/antibody complex formation, therefore  
 diagnosing a **flavivirus** infection in the subject (claimed). (I) is  
 useful as a vaccine for preventing **flavivirus** infection.

ADVANTAGE - (I) is easy to prepare and administer and is stable in  
 storage prior to use. (I) is essentially 100% successful in conferring  
 protective immunity in mammals after administering only a single dose. The  
 nucleic acid transcriptional unit is able to engender immunity to a  
**flavivirus** in a female mammal which can be transmitted its progeny to  
 the milk.

Dwg.0/8

L18 ANSWER 3 OF 3 WPIDS COPYRIGHT 2006 THE THOMSON CORP on STN

Full Text

AN 2000-072885 [06] WPIDS

CR 2003-058572 [05]

DNC C2000-020998

TI Novel nucleic acid for use in vaccines.

DC B04 D16

IN **CHANG, G J**

PA (USSH) US DEPT HEALTH & HUMAN SERVICES; (USSH) US CENTERS DISEASE CONTROL  
 & PREVENTION; (CHAN-I) CHANG G J

CYC 86

PI WO 9963095 A1 19991209 (200006)\* EN 58

RW: AT BE CH CY DE DK EA ES FI FR GB GH GM GR IE IT KE LS LU MC MW NL

OA PT SD SE SL SZ UG ZW

GD GE GH GM HR HU ID IL IN IS JP KE KG KP KR KZ LC LK LR LS LT LU  
 LV MD MG MK MN MW MX NO NZ PL PT RO RU SD SE SG SI SK SL TJ TM TR  
 TT UA UG US UZ VN YU ZA ZW  
 AU 9943296 A 19991220 (200021)  
 BR 9910830 A 20010213 (200114)  
 EP 1084252 A1 20010321 (200117) EN  
 R: DE FR GB NL  
 JP 2002517200 W 20020618 (200242) 63  
 US 2003022849 A1 20030130 (200311)  
 AU 778988 B2 20041223 (200510)  
 ADT WO 9963095 A1 WO 1999-US12298 19990603; AU 9943296 A AU 1999-43296  
 19990603; BR 9910830 A BR 1999-10830 19990603, WO 1999-US12298 19990603;  
 EP 1084252 A1 EP 1999-955295 19990603, WO 1999-US12298 19990603; JP  
 2002517200 W WO 1999-US12298 19990603, JP 2000-552289 19990603; US  
 2003022849 A1 Provisional US 1998-87908P 19980604, CIP of WO 1999-US12298  
 19990603, US 2001-826115 20010404, CIP of US 2001-701536 20010618; AU  
 778988 B2 AU 1999-43296 19990603  
 FDT AU 9943296 A Based on WO 9963095; BR 9910830 A Based on WO 9963095; EP  
 1084252 A1 Based on WO 9963095; JP 2002517200 W Based on WO 9963095; AU  
 778988 B2 Previous Publ. AU 9943296, Based on WO 9963095  
 PRAI US 1998-87908P 19980604; US 2001-826115 20010404;  
 US 2001-701536 20010618  
 AB WO 9963095 A UPAB: 20050928  
 NOVELTY - Nucleic acid molecule (I) comprises a transcription unit (TU)  
 for an immunogenic **flavivirus** antigen (Ag). When incorporated into a  
 host cell, TU directs synthesis of Ag.  
 DETAILED DESCRIPTION - INDEPENDENT CLAIMS are also included for the  
 following:  
 (1) host cells containing (I); and  
 (2) vaccines containing (I) plus a carrier.  
 ACTIVITY - Antiviral.  
 MECHANISM OF ACTION - Vaccine.  
 USE - (I) are used in vaccines to protect against **flavivirus**  
 infection. Also (not claimed) (I) can be used to produce Ag for analytical  
 or diagnostic applications. Plasmid pCBJEL-14 contains a fragment of  
 nucleic acid encoding the pre-M and E proteins of **Japanese**  
**encephalitis virus** (JEV) cloned into pCBamp. It was administered  
 intramuscularly (50-100 mu g) to 3-day old mice. After 7 weeks all animals  
 were seropositive for JEV and all were protected against subsequent  
 challenge by the mouse-adapted SA14 strain of JEV (contrast 40% survival  
 for animals inoculated with the commercial vaccine JE-VAX).  
 ADVANTAGE - (I) makes possible inexpensive and safe production of a  
 storage-stable vaccine that has minimal risk of causing adverse  
 immunological reactions to impurities. The vaccines elicit neutralizing  
 antibodies and protective immunity very effectively (i.e. 100%  
 protection), and since they contain only part of the viral genome they  
 can not cause infection in those manufacturing or receiving them. The  
 immunity conferred by the vaccine is transmitted to offspring through the  
 milk.  
 Dwg.0/8

=> d his

(FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006)

FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

E CHANG G J J/IN  
 E CHANG GWONG J J/IN  
 L1 2 S E4  
 E KONISHI E/AU  
 E KONISHI E/IN  
 L2 24958 S CMV OR CMV-IE  
 L3 3918 S L2 AND KOZAK  
 L4 3136 S L3 AND TERMINATION  
 L5 0 S L4 AND (POLY W A)  
 L6 760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR  
 L7 33 S L6 AND CMV/CLM  
 L8 11 S L7 AND AY<1999  
 L9 498 S L6 AND PCDNA3?  
 L10 9 S L9 AND AY<1999  
 L11 3 S L10 NOT L8  
 E SCHMALJOHN C S/IN  
 L12 12 S E4  
 L13 4840 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELLOW FEVER  
 L14 161 S L13 AND (PRM? AND E)  
 L15 82 S L14 AND (SIGNAL SEQUENCE)  
 L16 15 S L15 AND AY<1999

FILE 'WPIDS' ENTERED AT 22:19:11 ON 01 JUN 2006

E CHANG G J/IN  
 E CHANG G J J/IN

```

L18          3 S L17 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS O
=> s (flavivir? or dengue or japanese encephalitits viru or yellow fever virus)
      482 FLAVIVIR?
      493 DENGUE
     13912 JAPANESE
          0 ENCEPHALITITS
          9 VIRU
          0 JAPANESE ENCEPHALITITS VIRU
            (JAPANESE(W)ENCEPHALITITS(W)VIRU)
     37573 YELLOW
     5880 FEVER
     43600 VIRUS
          190 YELLOW FEVER VIRUS
            (YELLOW(W)FEVER(W)VIRUS)
L19          853 (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITITS VIRU OR YELLOW
              FEVER VIRUS)

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=> s 119 and (PrM? and E)
      513 PRM?
     2775088 E
L20          28 L19 AND (PRM? AND E)

```

```

=> s 120 and ay<1999

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SEARCH ENDED BY USER

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=>
SEARCH ENDED BY USER

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=> s 120 and CMV
      1674 CMV
L21          4 L20 AND CMV

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=> d 121,bib,ab,1-4

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L21  ANSWER 1 OF 4  WPIDS  COPYRIGHT 2006 THE THOMSON CORP on STN
Full Text
AN   2005-761339 [78]  WPIDS
DNC  C2005-232529
TI   Use of recombinant lentiviral vector for vaccination against infections by
      Flaviviridae, e.g. West Nile virus, dengue, yellow fever and hepatitis C.
DC   B04 C06 D16
IN   CHARNEAU, P; DESPRES, P; FRENKIEL, M P; TANGY, F; FRENKIEL, M
PA   (CNRS) CNRS CENT NAT RECH SCI; (INSP) INST PASTEUR; (CNRS) CENT NAT RECH
      SCI
CYC  111
PI   FR 2870126      A1 20051118 (200578)*      61
      WO 2005111221  A1 20051124 (200578)  EN
      RW: AT BE BG BW CH CY CZ DE DK EA EE ES FI FR GB GH GM GR HU IE IS IT
          KE LS LT LU MC MW MZ NA NL OA PL PT RO SD SE SI SK SL SZ TR TZ UG
          ZM ZW
      W:  AE AG AL AM AT AU AZ BA BB BG BR BW BY BZ CA CH CN CO CR CU CZ DE
          DK DM DZ EC EE EG ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG
          KM KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NA NG NI
          NO NZ OM PG PH PL PT RO RU SC SD SE SG SK SL SM SY TJ TM TN TR TT
          TZ UA UG US UZ VC VN YU ZA ZM ZW
ADT  FR 2870126 A1 FR 2004-5366 20040517; WO 2005111221 A1 WO 2005-IB1753
      20050516
PRAI FR 2004-5366      20040517
AB   FR 2870126 A UPAB: 20051205
      NOVELTY - Use of a recombinant lentiviral vector (A) to prepare an
      immunogenic composition for prevention and/or treatment of infections by
      Flaviviridae, where (A) includes a polynucleotide fragment (I) that
      encodes at least one protein (II) from a virus of the family
      Flaviviridae or an immunogenic peptide (IIa), of at least 8 amino acids,
      from (II).
      DETAILED DESCRIPTION - INDEPENDENT CLAIMS are also included for the
      following:
      (1) (A), As defined, that contains a polynucleotide that encodes at
      least one structural protein (or fragment) and optionally a non-structural
      protein (or fragment);
      (2) cells, preferably eukaryotic, modified by (A);
      (3) method for producing proteins of Flaviviridae and/or their
      immunogenic fragments or viral pseudoparticles, by culturing cells of (2);
      (4) method of screening for antiviral compounds using the cells of
      (2);
      (5) method for diagnosing infection by Flaviviridae in a biological
      fluid by detecting antibody-antigen complex formation with the cells of
      (2) or pseudoparticles of (3); and
      (6) kit for methods (4) and (5) that contains the cells of (2).

```



MECHANISM OF ACTION - Vaccine. Lentiviral vector TRIP Delta U3.**CMV**-Es(WNV), containing a 1.4 kb cDNA from West Nile virus (WNV), was used to immunize mice, at an intraperitoneal dose of 1 mu g. The anti-WNV antibody titer was 104 after 14 days and 2 plus or minus 105 after 23 days, with titer of antibodies that neutralize 90% of WNV loci of infections 10 and 20, respectively.

USE - (A) Are used to produce immunogenic compositions (vaccines) for treatment and/or prevention of **Flaviviridae** infections in humans and animals, particularly West Nile virus, **dengue**, yellow fever and hepatitis C.

Cells transformed with (A) are useful:

(i) for preparing proteins, or their immunogenic fragments, from **Flaviviridae**;

(ii) in screening for antiviral agents; and

(iii) to diagnose **Flaviviridae** infections.

ADVANTAGE - (A) Induces a strong response; particularly it targets antigen-presenting cells and (I) becomes integrated into the genome, ensuring stable expression in vivo, especially in dendritic cells, eliminating the need for repeated administrations. (A) Is non-replicative; non-tumorigenic; not species restricted and does not require adjuvants.  
Dwg.0/5

L21 ANSWER 2 OF 4 WPIDS COPYRIGHT 2006 THE THOMSON CORP on STN

Full Text

AN 2005-458565 [46] WPIDS

DNC C2005-139397

TI Novel polypeptide comprising fully human or humanized chimpanzee monoclonal antibody that binds or neutralizes **dengue** type 1, 2, 3, and/or 4 virus, useful for treatment and diagnosis of **dengue**.

DC B04 D16

IN LAI, C; PURCELL, R H

PA (USSH) US DEPT HEALTH & HUMAN SERVICES

CYC 108

PI WO 2005056600 A2 20050623 (200546)\* EN 121

RW: AT BE BG BW CH CY CZ DE DK EA EE ES FI FR GB GH GM GR HU IE IS IT  
KE LS LT LU MC MW MZ NA NL OA PL PT RO SD SE SI SK SL SZ TR TZ UG  
ZM ZW

W: AE AG AL AM AT AU AZ BA BB BG BR BW BY BZ CA CH CN CO CR CU CZ DE  
DK DM DZ EC EE EG ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG  
KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NA NI NO NZ  
OM PG PH PL PT RO RU SC SD SE SG SK SL SY TJ TM TN TR TT TZ UA UG  
US UZ VC VN YU ZA ZM ZW

ADT WO 2005056600 A2 WO 2004-US40674 20041203

PRAI US 2004-624261P 20041101; US 2003-528161P 20031208;

US 2004-541676P 20040204; US 2004-552528P 20040312;

US 2004-574492P 20040526

AB WO2005056600 A UPAB: 20050720

NOVELTY - A substantially pure polypeptide (I) comprising (a) a fully human or humanized chimpanzee monoclonal antibody that binds or neutralizes **dengue** type 1, 2, 3, and/or 4 virus, (b) a monoclonal antibody that binds the antigen to which monoclonal antibody 5H2 (ATCC Accession No. PTA-5662) binds, or (c) a monoclonal antibody that binds the antigen to which monoclonal antibody 1A5 (ATCC Accession No. PTA-6265) binds, is new.

DETAILED DESCRIPTION - INDEPENDENT CLAIMS are also included for the following:

(1) an isolated nucleic acid (II) comprising a nucleotide sequence encoding (I);

(2) a host cell including a vector comprising (II);

(3) a pharmaceutical preparation (III) comprising a carrier and (I);

(4) a diagnostic preparation (IV) comprising a carrier and (I);

(5) humanized IgG1 5H2 plasmid deposited with ATCC as ATCC Accession No. PTA-5662;

(6) humanized IgG1 1A5 plasmid deposited with ATCC as ATCC Accession No. PTA-6265; and

(7) pFab **CMV**-dhfr vector for expression of any full-length IgG1 deposited with ATCC as PTA-5662.

ACTIVITY - Virucide.

The neutralization of DENV-2/DENV-4 chimeras by Fab 1A5 was determined. DENV-2/DENV-4 chimeras composed of the parental DENV-2 NGB C-**prM-E** sequence of the variant C-**prM-E** sequence specifying the His317Gln or Gly106Val substitution present in NGB-V1 and NGB-V2, were prepared. Fab 1A5 neutralized the chimeric DENV-2(NGB-P)/DENV-4 at a PRNT50 titer of 0.64 micro g/ml. The chimera containing Gly106Val had a PRNT50 titer of greater than 70 micro g/ml and the chimera containing His317Gln had a PRNT50 titer of 31.7 micro g/ml, similar to that measured for NGB-V2 and NGB-V1, respectively.

MECHANISM OF ACTION - Viral neutralization.

USE - (I) is useful as pharmaceutical preparation and a diagnostic preparation for treatment and diagnosis of **dengue** virus disease. (III) is useful in treatment of and prophylaxis against **dengue** virus disease, which involves administering (III) to the patient. (IV) is useful in the

the patient, and detecting binding of (I) as a determination of the presence of **dengue** virus disease. (IV) is useful for detecting the presence of **dengue** virus in a biological sample, which involves contacting the sample with (IV) and assaying binding of (I) as a determination of the **dengue** virus (all claimed).

Dwg.0/18

L21 ANSWER 3 OF 4 WPIDS COPYRIGHT 2006 THE THOMSON CORP on STN

Full Text

AN 2005-031713 [03] WPIDS

DNC C2005-010142

TI New packaging construct comprising a regulatable promoter operably linked to a nucleotide sequence encoding a **flavivirus** structural protein translation product, for regulatable expression of the structural proteins in animal cell.

DC B04 D16

IN KHROMYKH, A A; KHROMYKH, A

PA (UYQU) UNIV QUEENSLAND

CYC 109

PI WO 2004108936 A1 20041216 (200503)\* EN 53

RW: AT BE BG BW CH CY CZ DE DK EA EE ES FI FR GB GH GM GR HU IE IT KE

LS LU MC MW MZ NA NL OA PL PT RO SD SE SI SK SL SZ TR TZ UG ZM ZW

W: AE AG AL AM AT AU AZ BA BB BG BR BW BY BZ CA CH CN CO CR CU CZ DE

DK DM DZ EC EE EG ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG

KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NA NI NO NZ

OM PG PH PL PT RO RU SC SD SE SG SK SL SY TJ TM TN TR TT TZ UA UG

US UZ VC VN YU ZA ZM ZW

AU 2004245578 A1 20041216 (200604)

EP 1633877 A1 20060315 (200620) EN

R: AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LI LU MC NL PL

PT RO SE SI SK TR

ADT WO 2004108936 A1 WO 2004-AU752 20040607; AU 2004245578 A1 AU 2004-245578

20040607; EP 1633877 A1 EP 2004-736184 20040607, WO 2004-AU752 20040607

FDT AU 2004245578 A1 Based on WO 2004108936; EP 1633877 A1 Based on WO 2004108936

PRAI AU 2003-902842 20030606

AB WO2004108936 A UPAB: 20050112

NOVELTY - A packaging construct for regulatable expression of **flavivirus** structural proteins in an animal cell comprising a regulatable promoter operably linked to a nucleotide sequence encoding a **flavivirus** structural protein translation product that comprises C protein, **prM** protein and **E** protein, is new.

DETAILED DESCRIPTION - INDEPENDENT CLAIMS are included for the following:

(1) a packaging cell comprising the packaging construct, and/or a tetracycline transactivator construct;

(2) a **flaviviral** packaging system comprising the packaging construct; and a **flaviviral** expression construct comprising a **flaviviral** replicon, a heterologous nucleic acid, and a promoter operably linked to the replicon;

(3) a packaging cell comprising the **flaviviral** packaging system;

(4) producing **flavivirus** VLPs;

(5) **flaviviral** VLPs produced by the method;

(6) an immunotherapeutic composition comprising the VLPs and a diluent or excipient;

(7) producing a recombinant protein; and

(8) immunizing an animal.

ACTIVITY - None given.

MECHANISM OF ACTION - Vaccine.

USE - The packaging construct is useful for regulatable expression of **flavivirus** structural proteins in an animal cell (claimed).

Dwg.0/8

L21 ANSWER 4 OF 4 WPIDS COPYRIGHT 2006 THE THOMSON CORP on STN

Full Text

AN 2004-653420 [63] WPIDS

DNC C2004-233874

TI New vector comprising gene promoting and suppressing cassettes, useful for regulating gene expression and for preventing or treating **Dengue** virus infection.

DC B04 D16

IN MOHAPATRA, S S; ZHANG, W

PA (UYSF-N) UNIV SOUTH FLORIDA

CYC 108

PI WO 2004076664 A2 20040910 (200463)\* EN 53

RW: AT BE BG BW CH CY CZ DE DK EA EE ES FI FR GB GH GM GR HU IE IT KE

LS LU MC MW MZ NL OA PT RO SD SE SI SK SL SZ TR TZ UG ZM ZW

W: AE AG AL AM AT AU AZ BA BB BG BR BW BY BZ CA CH CN CO CR CU CZ DE

DK DM DZ EC EE EG ES FI GB GD GE GH GM HR HU ID IL IN IS JP KE KG

KP KR KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NA NI NO NZ

OM PG PH PL PT RO RU SC SD SE SG SK SL SY TJ TM TN TR TT TZ UA UG

US UZ VC VN YU ZA ZM ZW

PRAI US 2003-320108P 20030415; US 2003-319964P 20030221  
 AB WO2004076664 A UPAB: 20041001  
 NOVELTY - A vector comprising expression cassettes, which have at least one gene promoting and suppressing cassette comprising a first and a second polynucleotide operably linked to a first and a second promoter sequence, respectively, where the second polynucleotide encodes a short interfering RNA (siRNA) molecule that reduces expression of a target gene by RNA interference, is new.  
 DETAILED DESCRIPTION - INDEPENDENT CLAIMS are also included for:  
 (1) modulating the expression of multiple genes within a host;  
 (2) inhibiting the expression of **Dengue** virus genes within a host;  
 (3) a pharmaceutical composition comprising the novel vector and a pharmaceutical carrier; and  
 (4) producing a vector for modulating the expression of multiple genes or for inhibiting the expression of **Dengue** virus.  
 ACTIVITY - Virucide.  
 No biological data given.  
 MECHANISM OF ACTION - Gene Therapy.  
 USE - The vector, methods, and composition are useful for regulating gene expression and for preventing or treating **Dengue** virus infection.  
 Dwg.0/10

=> d

L21 ANSWER 1 OF 4 WPIDS COPYRIGHT 2006 THE THOMSON CORP on STN  
 Full Text  
 AN 2005-761339 [78] WPIDS  
 DNC C2005-232529  
 TI Use of recombinant lentiviral vector for vaccination against infections by **Flaviviridae**, e.g. West Nile virus, **dengue**, yellow fever and hepatitis C.  
 DC B04 C06 D16  
 IN CHARNEAU, P; DESPRES, P; FRENKIEL, M P; TANGY, F; FRENKIEL, M  
 PA (CNRS) CNRS CENT NAT RECH SCI; (INSP) INST PASTEUR; (CNRS) CENT NAT RECH SCI  
 CYC 111  
 PI FR 2870126 A1 20051118 (200578)\* 61 A61K048-00  
 WO 2005111221 A1 20051124 (200578) EN C12N015-867  
 RW: AT BE BG BW CH CY CZ DE DK EA EE ES FI FR GB GH GM GR HU IE IS IT  
 KE LS LT LU MC MW MZ NA NL OA PL PT RO SD SE SI SK SL SZ TR TZ UG  
 ZM ZW  
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 NO NZ OM PG PH PL PT RO RU SC SD SE SG SK SL SM SY TJ TM TN TR TT  
 TZ UA UG US UZ VC VN YU ZA ZM ZW  
 ADT FR 2870126 A1 FR 2004-5366 20040517; WO 2005111221 A1 WO 2005-IB1753  
 20050516  
 PRAI FR 2004-5366 20040517  
 IC ICM A61K048-00; C12N015-867  
 ICS A61K039-12; A61P031-14; C07K014-18; C12N005-10; C12N015-40;  
 C12N015-86; C12Q001-02; C12Q001-70; G01N033-569

=> d his

(FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006)

FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

E CHANG G J J/IN  
 E CHANG GWONG J J/IN  
 L1 2 S E4  
 E KONISHI E/AU  
 E KONISHI E/IN  
 L2 24958 S CMV OR CMV-IE  
 L3 3918 S L2 AND KOZAK  
 L4 3136 S L3 AND TERMINATION  
 L5 0 S L4 AND (POLY W A)  
 L6 760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR  
 L7 33 S L6 AND CMV/CLM  
 L8 11 S L7 AND AY<1999  
 L9 498 S L6 AND PCDNA3?  
 L10 9 S L9 AND AY<1999  
 L11 3 S L10 NOT L8  
 E SCHMALJOHN C S/IN  
 L12 12 S E4  
 L13 4840 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELLOW FEVER  
 L14 161 S L13 AND (PRM? AND E)  
 L15 82 S L14 AND (SIGNAL SEQUENCE)  
 L16 15 S L15 AND AY<1999

FILE 'WPIDS' ENTERED AT 22:19:11 ON 01 JUN 2006

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      E CHANG G J J/IN
L17      121 S E2
L18      3 S L17 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS O
L19      853 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITITS VIRU OR YELLOW
L20      28 S L19 AND (PRM? AND E)
L21      4 S L20 AND CMV

```

=> file medline

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	99.12	307.85

FILE 'MEDLINE' ENTERED AT 22:28:38 ON 01 JUN 2006

FILE LAST UPDATED: 1 JUN 2006 (20060601/UP). FILE COVERS 1950 TO DATE.

On December 11, 2005, the 2006 MeSH terms were loaded.

The MEDLINE reload for 2006 is now (26 Feb.) available. For details on the 2006 reload, enter HELP RLOAD at an arrow prompt (=>). See also:

<http://www.nlm.nih.gov/mesh/>  
[http://www.nlm.nih.gov/pubs/techbull/nd04/nd04\\_mesh.html](http://www.nlm.nih.gov/pubs/techbull/nd04/nd04_mesh.html)  
[http://www.nlm.nih.gov/pubs/techbull/nd05/nd05\\_med\\_data\\_changes.html](http://www.nlm.nih.gov/pubs/techbull/nd05/nd05_med_data_changes.html)  
[http://www.nlm.nih.gov/pubs/techbull/nd05/nd05\\_2006\\_MeSH.html](http://www.nlm.nih.gov/pubs/techbull/nd05/nd05_2006_MeSH.html)

OLDMEDLINE is covered back to 1950.

MEDLINE thesauri in the /CN, /CT, and /MN fields incorporate the MeSH 2006 vocabulary.

This file contains CAS Registry Numbers for easy and accurate substance identification.

=> e chang g j j/au

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E1      9      CHANG G H/AU
E2      50     CHANG G J/AU
E3      0 --> CHANG G J J/AU
E4      2      CHANG G K/AU
E5      23     CHANG G L/AU
E6      1      CHANG G M/AU
E7      7      CHANG G N/AU
E8      14     CHANG G Q/AU
E9      2      CHANG G R/AU
E10     4      CHANG G S/AU
E11     28     CHANG G T/AU
E12     3      CHANG G T G/AU

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=> s e2

```

L22      50 "CHANG G J"/AU

```

=> s l22 and (flavivir? or dengue or yellow fever or japanese encephalitis)

```

      3719 FLAVIVIR?
      5142 DENGUE
      21763 YELLOW
      107428 FEVER
      3054 YELLOW FEVER
          (YELLOW(W)FEVER)
      50156 JAPANESE
      28653 ENCEPHALITIS
      2119 JAPANESE ENCEPHALITIS
          (JAPANESE(W)ENCEPHALITIS)

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L23      23 L22 AND (FLAVIVIR? OR DENGUE OR YELLOW FEVER OR JAPANESE ENCEPHA
          LITIS)

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=> s l23 and py<2000

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      12412454 PY<2000
          (PY<20000000)

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L24      16 L23 AND PY<2000

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=> d l24,cbib,ab,1-16

L24 ANSWER 1 OF 16 MEDLINE on STN

2004593406. PubMed ID: 15566752. Detection of **yellow fever** virus by polymerase chain reaction. Brown T M; **Chang G J**; Cropp C B; Robbins K E; Tsai T F. (Division of Vector-Borne Infectious Diseases, National Centers for Infectious Diseases, Centers for Disease Control, P.O. Box 2087, Fort Collins, CO 80522, USA. ) Clinical and diagnostic virology, (1994 Feb) Vol. 2, No. 1, pp. 41-51. Journal code: 9309653. ISSN: 0928-0197. Pub. country: Netherlands. Language: English.

AB BACKGROUND: **Yellow fever** virus continues to cause major epidemics. A

in order to implement emergency immunization campaigns. OBJECTIVES: To identify YFV envelope protein gene fragments, construct a polymerase chain reaction (PCR) assay and test its utility in identifying viruses isolated from laboratory and clinical specimens. STUDY DESIGN: YFV RNA was transcribed with reverse transcriptase and the cDNA amplified by PCR using primers encoding a portion of the viral envelope protein gene. The identity of the 482 bp amplified product was confirmed by restriction enzyme analysis and by dot blot hybridization with a labelled oligonucleotide probe. The assay was tested for sensitivity and specificity on isolates from South America and Africa. Detection limits were determined using different probe labels. PCR inhibitory effects were analyzed with laboratory and clinical specimens. RESULTS: The assay was specific for YFV and did not detect any of 15 other **flaviviruses**. The amplified region was conserved among all 32 South American and African isolates tested. Four strains from Africa did not hybridize with the probe, indicating sequence divergence in the envelope protein gene. Samples containing 30 pfu of virus were detected by visual inspection of the ethidium bromide stained 482 bp DNA amplicon and 10 pfu were detected with a digoxigenin labelled probe. Inhibitory effects of human serum on the PCR were overcome by diluting samples 4-fold in buffer. Viral neutralizing antibody in experimental samples did not affect the sensitivity of detection. **Yellow fever** virus in serum from experimentally infected Cynomolgus monkeys (10(3.7)-10(7.0) pfu/0.1 ml) was detected with signal intensities corresponding to the amount of virus in the sample. When YFV was added to normal human serum and held at 27 degrees C and 80% humidity, the RNA could be detected for up to 3 weeks in samples that had no infectious virus. CONCLUSIONS: A PCR assay was constructed which detected YFV RNA in isolates from patients infected in South America and Africa. This assay is specific for YFV but some African strains were not detected. More clinical samples should be tested.

L24 ANSWER 2 OF 16 MEDLINE on STN

1998284223. PubMed ID: 9621203. Molecular and epidemiologic analysis of **dengue** virus isolates from Somalia. Kanesa-athan N; **Chang G J**; Smoak B L; Magill A; Burrous M J; Hoke C H Jr. (Walter Reed Army Institute of Research, Washington, DC 20307-5100, USA.. maj.niranjan.kanesa@wrsmtt-cmail-army.mil) . Emerging infectious diseases, (1998 Apr-Jun) Vol. 4, No. 2, pp. 299-303. Journal code: 9508155. ISSN: 1080-6040. Pub. country: United States. Language: English.

AB Nucleotide sequence analysis was performed on 14 **dengue** virus isolates (13 **dengue**-2 viruses and 1 **dengue**-3 virus) recovered from febrile soldiers in Somalia in 1993. The **dengue**-2 viruses were most closely related to **dengue**-2 virus recovered in Somalia in 1984. However, differences in nucleotide sequence (0.35% to 1.35%) were evident among the 1993 isolates. These differences were closely associated with the geographic location of the infection as well as with different times of infection at the same location. Genetic difference between strains was not associated with differences in clinical features. Molecular analysis of **dengue** viruses is a useful adjunct to epidemiologic investigation of their distribution over distance and time.

L24 ANSWER 3 OF 16 MEDLINE on STN

1998080391. PubMed ID: 9420202. Phylogeny of the genus **Flavivirus**. Kuno G; **Chang G J**; Tsuchiya K R; Karabatsos N; Cropp C B. (Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado 80522-2087, USA.. GOKI@CDC.GOV) . Journal of virology, (1998 Jan) Vol. 72, No. 1, pp. 73-83. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB We undertook a comprehensive phylogenetic study to establish the genetic relationship among the viruses of the genus **Flavivirus** and to compare the classification based on molecular phylogeny with the existing serologic method. By using a combination of quantitative definitions (bootstrap support level and the pairwise nucleotide sequence identity), the viruses could be classified into clusters, clades, and species. Our phylogenetic study revealed for the first time that from the putative ancestor two branches, non-vector and vector-borne virus clusters, evolved and from the latter cluster emerged tick-borne and mosquito-borne virus clusters. Provided that the theory of arthropod association being an acquired trait was correct, pairwise nucleotide sequence identity among these three clusters provided supporting data for a possibility that the non-vector cluster evolved first, followed by the separation of tick-borne and mosquito-borne virus clusters in that order. Clades established in our study correlated significantly with existing antigenic complexes. We also resolved many of the past taxonomic problems by establishing phylogenetic relationships of the antigenically unclassified viruses with the well-established viruses and by identifying synonymous viruses.

L24 ANSWER 4 OF 16 MEDLINE on STN

1998063965. PubMed ID: 9402373. Imported **yellow fever** in a United States citizen. McFarland J M; Baddour L M; Nelson J E; Elkins S K; Craven R B; Cropp B C; **Chang G J**; Grindstaff A D; Craig A S; Smith R J.

Tennessee Medical Center at Knoxville, 37920-6999, USA. ) Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, (1997 Nov) Vol. 25, No. 5, pp. 1143-7. Journal code: 9203213. ISSN: 1058-4838. Pub. country: United States. Language: English.

AB The last imported case of **yellow fever** seen in this country was in 1924. We report a case of **yellow fever** acquired by an American tourist who visited the jungles of Brazil along the Rio Negro and Amazon Rivers. The patient died 6 days after hospital admission and 10 days after his first symptoms appeared. **Yellow fever** virus was recovered from clinical specimens, and the isolate was genetically similar to the E genotype IIB of South American **yellow fever** viruses. This patient's illness represents a case of vaccine-preventable death since he failed to be immunized with a recommended preexposure **yellow fever** vaccine.

L24 ANSWER 5 OF 16 MEDLINE on STN

97288308. PubMed ID: 9143286. Construction of infectious cDNA clones for **dengue 2** virus: strain 16681 and its attenuated vaccine derivative, strain PDK-53. Kinney R M; Butrapet S; **Chang G J**; Tsuchiya K R; Roehrig J T; Bhamarapravati N; Gubler D J. (Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado, USA.. rmkl@cdc.gov) . Virology, (1997 Apr 14) Vol. 230, No. 2, pp. 300-8. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB We identified nine nucleotide differences between the genomes of **dengue-2** (DEN-2) 16681 virus and its vaccine derivative, strain PDK-53. These included a C-to-T (16681-to-PDK-53) mutation at nucleotide position 57 of the 5'-untranslated region, three silent mutations, and substitutions prM-29 Asp to Val, NS1-53 Gly to Asp, NS2A-181 Leu to Phe, NS3-250 Glu to Val, and NS4A-75 Gly to Ala. Unpassaged PDK-53 vaccine contained two genetic variants as a result of partial mutation at NS3-250. We constructed infectious cDNA clones for 16681 virus and each of the two PDK-53 variants. DEN-2 16681 clone-derived viruses were identical to the 16681 virus in plaque size and replication in LLC-MK2 cells, replication in C6/36 cells, E and prM epitopes, and neurovirulence for suckling mice. PDK-53 virus and both clone-derived PDK-53 variants were attenuated in mice. However, the variant containing NS3-250-Glu was less temperature sensitive and replicated better in C6/36 cells than did PDK-53 virus. The variant containing NS3-250-Val had smaller, more diffuse plaques, decreased replication, and increased temperature sensitivity in LLC-MK2 cells relative to PDK-53 virus. Both PDK-53 virus and the NS3-250-Val variant replicated poorly in C6/36 cells relative to 16681 virus. Unpassaged PDK-53 vaccine virus and the virus passaged once in LLC-MK2 cells had genomes of identical sequence, including the mixed NS3-250-Glu/Val locus. Although the NS3-250-Val mutation clearly affected virus replication in vitro, it was not a major determinant of attenuation for PDK-53 virus in suckling mice.

L24 ANSWER 6 OF 16 MEDLINE on STN

95363991. PubMed ID: 7637022. Nucleotide sequence variation of the envelope protein gene identifies two distinct genotypes of **yellow fever** virus. **Chang G J**; Cropp B C; Kinney R M; Trent D W; Gubler D J. (Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado 80522, USA. ) Journal of virology, (1995 Sep) Vol. 69, No. 9, pp. 5773-80. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB The evolution of **yellow fever** virus over 67 years was investigated by comparing the nucleotide sequences of the envelope (E) protein genes of 20 viruses isolated in Africa, the Caribbean, and South America. Uniformly weighted parsimony algorithm analysis defined two major evolutionary **yellow fever** virus lineages designated E genotypes I and II. E genotype I contained viruses isolated from East and Central Africa. E genotype II viruses were divided into two sublineages: IIA viruses from West Africa and IIB viruses from America, except for a 1979 virus isolated from Trinidad (TRINID79A). Unique signature patterns were identified at 111 nucleotide and 12 amino acid positions within the **yellow fever** virus E gene by signature pattern analysis. **Yellow fever** viruses from East and Central Africa contained unique signatures at 60 nucleotide and five amino acid positions, those from West Africa contained unique signatures at 25 nucleotide and two amino acid positions, and viruses from America contained such signatures at 30 nucleotide and five amino acid positions in the E gene. The dissemination of **yellow fever** viruses from Africa to the Americas is supported by the close genetic relatedness of genotype IIA and IIB viruses and genetic evidence of a possible second introduction of **yellow fever** virus from West Africa, as illustrated by the TRINID79A virus isolate. The E protein genes of American IIB **yellow fever** viruses had higher frequencies of amino acid substitutions than did genes of **yellow fever** viruses of genotypes I and IIA on the basis of comparisons with a consensus amino acid sequence for the **yellow fever** E gene. The great variation in the E proteins of American **yellow fever** virus probably results from positive selection imposed by virus interaction with different species of mosquitoes or nonhuman

L24 ANSWER 7 OF 16 MEDLINE on STN

95156016. PubMed ID: 7852952. Homologous and heterologous neutralization antibody responses after immunization with **Japanese encephalitis** vaccine among Taiwan children. Ku C C; King C C; Lin C Y; Hsu H C; Chen L Y; Yueh Y Y; **Chang G J**. (Institute of Public Health, National Taiwan University, Taipei, Republic of China. ) Journal of medical virology, (1994 Oct) Vol. 44, No. 2, pp. 122-31. Journal code: 7705876. ISSN: 0146-6615. Pub. country: United States. Language: English.

AB Because 21 immunized children (13%) among the 162 confirmed **Japanese encephalitis** (JE) cases during 1986-1991 occurred in Taiwan, we collected 320 serum samples from Taiwan children aged 15-31 and 27-44 months immediately before the 1st dose (n = 41) and 1-3 months after the 2nd dose (n = 78, 27 pairs), and immediately before (n = 58) and 1-3 months after the 3rd dose (n = 143, 44 pairs) to determine neutralization antibody (Nt Ab) against the Nakayama (N) and Beijing-1 (B) strains and two Taiwan wild type JE viruses (JEV): CC-27 and CH-1392. Our Nt results showed that (1) B vaccine stimulated a better homologous Ab response than N vaccine for Nt Ab seropositivity rate (NASR), produced a higher level of Nt titer after the primary immunization [2 doses = 100% vs. 91%, geometric mean titer (GMT) = 115 vs. 22], had a greater booster effect (3 doses: 100% vs. 95%; GMT = 320 vs 33), and showed a better capability to neutralize two local Taiwan JEV strains, particularly only after 3 doses (ave. NASR for B vs. N = 90% vs. 10%; and GMT for B vs. N = 154 vs. 1); (2) the two wild type JEV strains had different plaque morphology and antigenic variation and the CC-27 strain was not neutralized as well as the CH-1392 strain after 3 doses of vaccine (BBB or NNN or NNB); and (3) 30% of the children had lost JEV Nt Ab one year after the 2nd dose of N vaccine and natural infection with JE virus did occur among those children after immunization. In conclusion, (1) three doses of mouse-brain vaccine are the minimum requirement to protect children against the local Taiwan JEV-, (2) the best strain for a JE vaccine depends on level of Nt Ab it induced, the molecular epidemiology and antigenic variation of the JEV in each local area; and (3) future vaccine must produce better B- and T-cell memory.

L24 ANSWER 8 OF 16 MEDLINE on STN

95146982. PubMed ID: 7844560. Molecular basis of attenuation of neurovirulence of wild-type **Japanese encephalitis** virus strain SA14. Ni H; **Chang G J**; Xie H; Trent D W; Barrett A D. (Department of Pathology F-05, University of Texas Medical Branch, Galveston 77555-0605. ) The Journal of general virology, (1995 Feb) Vol. 76 ( Pt 2), pp. 409-13. Journal code: 0077340. ISSN: 0022-1317. Pub. country: ENGLAND: United Kingdom. Language: English.

AB To identify the molecular determinants for attenuation of wild-type **Japanese encephalitis** (JE) virus strain SA14, the RNA genome of wild-type strain SA14 and its attenuated vaccine virus SA14-2-8 were reverse transcribed, amplified by PCR and sequenced. Comparison of the nucleotide sequence of SA14-2-8 vaccine virus with virulent parent SA14 virus and with two other attenuated vaccine viruses derived from SA14 virus (SA14-14-2/PHK and SA14-14-2/PDK) revealed only seven amino acids in the virulent parent SA14 had been substituted in all three attenuated vaccines. Four were in the envelope (E) protein (E-138, E-176, E-315 and E-439), one in non-structural protein 2B (NS2B-63), one in NS3 (NS3-105), and one in NS4B (NS4B-106). The substitutions at E-315 and E-439 arose due to correction of the SA14/CDC sequence published previously by Nitayaphan et al. (Virology 177, 541-552, 1990). The mutations in NS2B and NS3 are in functional domains of the trypsin-like serine protease. Attenuation of SA14 virus may therefore, in part, be due to alterations in viral protease activity, which could affect replication of the virus.

L24 ANSWER 9 OF 16 MEDLINE on STN

95076700. PubMed ID: 7985409. Nucleotide sequence analysis of the structural gene coding region of the pestivirus border disease virus. Sullivan D G; **Chang G J**; Trent D W; Akkina R K. (Department of Pathology, Colorado State University, Fort Collins 80523. ) Virus research, (1994 Sep) Vol. 33, No. 3, pp. 219-28. Journal code: 8410979. ISSN: 0168-1702. Pub. country: Netherlands. Language: English.

AB Border disease virus (BDV) of sheep, an important ovine pathogen, is serologically related to the two other well characterized members of the Pestivirus genus of the **Flaviviridae** family, namely bovine viral diarrhea virus (BVDV) and hog cholera virus (HoCV). To determine its genetic relationship to BVDV and HoCV, the genome of BDV strain, BD-78 encompassing the 5' untranslated region (UTR) and structural gene coding region was molecularly cloned and the nucleotide sequence determined. The sequenced region of 3,567 nucleotides contained one open reading frame encoding 1063 amino acids. The nucleotide and amino acid sequences of BD-78 were compared with those of two BVDV strains NADL and SD-1, and the Alfort and Brescia strains of HoCV. The overall nucleotide sequence homologies of the region sequenced of BD-78 are 68.3% with BVDV-NADL, 67.8% with BVDV-SD-1, 69.0% with HoCV-Brescia, and 65.8% with HoCV-Alfort. The overall amino acid sequence homologies of BD-78 are 76.1% with NADL,

conserved nucleotide and amino acid sequences between BD-78 and the other pestiviruses are in the 5' UTR and the capsid protein coding region (p14), whereas the most divergent sequences are in the E2 coding region. These findings suggest that BDV is a unique virus in the Pestivirus genus.

L24 ANSWER 10 OF 16 MEDLINE on STN

94267439. PubMed ID: 8207417. Comparison of nucleotide and deduced amino acid sequence of the 5' non-coding region and structural protein genes of the wild-type **Japanese encephalitis** virus strain SA14 and its attenuated vaccine derivatives. Ni H; Burns N J; **Chang G J**; Zhang M J; Wills M R; Trent D W; Sanders P G; Barrett A D. (Department of Pathology F-05, University of Texas Medical Branch, Galveston 77555-0605. ) The Journal of general virology, (1994 Jun) Vol. 75 ( Pt 6), pp. 1505-10. Journal code: 0077340. ISSN: 0022-1317. Pub. country: ENGLAND: United Kingdom. Language: English.

AB Nucleotide sequences of the 5' non-coding region and the structural protein genes of the live, attenuated **Japanese encephalitis** vaccine virus strains SA14-2-8 and SA14-5-3 and the wild-type parental strain SA14/USA were determined. SA14-2-8 differed from SA14/USA by 13 nucleotides and eight amino acids whereas SA14-5-3 differed from SA14/USA by 15 nucleotides and eight amino acids. A comparison of the 5' non-coding region and amino acid sequences of the structural proteins of these two attenuated vaccine strains and of vaccine strains SA14-14-2/PHK and SA14-14-2/PDK with three sequences of their wild-type parent SA14 virus was performed. This revealed only two common amino acid substitutions at positions 138 and 176 in the envelope (E) protein. The substitution at E138 was predicted to cause a change in the secondary structure of the E protein. These two amino acid substitutions in the E protein may contribute to attenuation of the **Japanese encephalitis** vaccine viruses.

L24 ANSWER 11 OF 16 MEDLINE on STN

94201380. PubMed ID: 7512096. An integrated target sequence and signal amplification assay, reverse transcriptase-PCR-enzyme-linked immunosorbent assay, to detect and characterize **flaviviruses**. **Chang G J**; Trent D W; Vorndam A V; Vergne E; Kinney R M; Mitchell C J. (Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado 80522. ) Journal of clinical microbiology, (1994 Feb) Vol. 32, No. 2, pp. 477-83. Journal code: 7505564. ISSN: 0095-1137. Pub. country: United States. Language: English.

AB We previously described a reverse transcriptase-PCR using **flavivirus** genus-conserved and virus species-specific amplimers (D. W. Trent and G. J. Chang, p. 355-371, in Y. Becker and C. Darai; ed., Frontiers of Virology, vol. 1, 1992). Target amplification was improved by redesigning the amplimers, and a sensitive enzyme-linked immunosorbent assay (ELISA) technique has been developed to detect amplified digoxigenin (DIG)-modified DNA. A single biotin motif and multiple DIG motifs were incorporated into each amplicon, which permitted amplicon capture by a biotin-streptavidin interaction and detection with DIG-specific antiserum in a colorimetric ELISA. We evaluated the utility of this assay for detecting St. Louis encephalitis (SLE) viral RNA in infected mosquitoes and **dengue** viral RNA in human serum specimens. The reverse transcriptase-PCR-ELISA was as sensitive as isolation of SLE virus by cell culture in detecting SLE viral RNA in infected mosquitoes. The test was 89% specific and 95 to 100% sensitive for identification of **dengue** viral RNA in serum specimens compared with isolation of virus by Aedes albopictus C6/36 cell culture and identification by the indirect immunofluorescence assay.

L24 ANSWER 12 OF 16 MEDLINE on STN

94025587. PubMed ID: 8105605. A comparison of the nucleotide sequences of eastern and western equine encephalomyelitis viruses with those of other alphaviruses and related RNA viruses. Weaver S C; Hagenbaugh A; Bellow L A; Netesov S V; Volchkov V E; **Chang G J**; Clarke D K; Gousset L; Scott T W; Trent D W; +. (Department of Biology, University of California, San Diego, La Jolla 92093. ) Virology, (1993 Nov) Vol. 197, No. 1, pp. 375-90. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB The complete nucleotide sequence of a 1982 Florida strain of eastern equine encephalomyelitis (EEE) virus, and partial sequence of the nonstructural protein genes of western equine encephalomyelitis (WEE) virus, were determined. The EEE virus genome was 11,678 nucleotides in length, excluding the cap nucleotide and poly(A) tail, and the nucleotide composition was 28% A, 24% G, 25% C, and 23% U. The organization of both EEE and WEE virus genomes was like that of other alphaviruses and included a termination codon between the nsP3 and nsP4 genes. Codon usage for 10 of 20 amino acids was nonrandom in the EEE genome, and dinucleotide CpG-containing codons were underutilized in both genomes. The slight CpG deficiency was similar to that seen in other alphaviruses and plant viruses in the alphavirus-like group, but less than that of poliovirus and **yellow fever** virus. This slight deficiency may reflect adaptation for replication in both CpG-deficient vertebrates, as well as insects which do



protein amino acid sequences indicated that alphaviruses evolved from a common ancestor which existed a few thousand years ago. An intercontinental introduction of an ancestral virus from the Old to New World, or vice versa, probably resulted in two main extant groups: one includes New World (EEE and Venezuelan equine encephalitis) viruses, while the other includes Old World (Sindbis, Middelburg, O'nyong-nyong, Ross River, and Semliki Forest) viruses. The position of WEE virus in the phylogenetic trees indicated that, in addition to its capsid gene (C. S. Hahn et al. (1988) Proc. Natl. Acad. Sci. USA 85, 5997-6001), WEE virus acquired its nonstructural genes from an EEE-like ancestor during recombination.

L24 ANSWER 13 OF 16 MEDLINE on STN

94025568. PubMed ID: 8212556. Phylogenetic relationships of **dengue-2** viruses. Lewis J A; **Chang G J**; Lanciotti R S; Kinney R M; Mayer L W; Trent D W. (Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado 80522. ) Virology, (1993 Nov) Vol. 197, No. 1, pp. 216-24. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB RNA oligonucleotide fingerprinting studies on a large number of virus isolates previously demonstrated considerable genetic variation in isolates of **dengue** (DEN)-2 serotype. We report the entire envelope (E) glycoprotein gene and deduced amino acid sequences of 16 DEN-2 viruses and the phylogenetic relationships of these, plus 17 additional published DEN E gene sequences. Comparison of DEN-2 E glycoprotein gene sequences revealed base substitutions scattered throughout the entire gene with as much as 22% sequence divergence. Aligned E glycoprotein amino acid sequences revealed the viruses differed by as much as 10%. There appeared to be constraints on the overall structure of the E protein to maintain biological function. Clusters of amino acid substitutions were present in the hydrophobic membrane anchor region at the carboxyl terminal end of the protein. Maximum parsimony analysis of the E gene sequences allowed construction of a phylogram indicating evolutionary relationships of the virus isolates within the DEN-2 serotype. Five genetic subtypes were identified. Phylogenetic relationships of the DEN-2 serotype and other **flaviviruses** based on E protein sequences reflected traditional antigenic and serologic classifications.

L24 ANSWER 14 OF 16 MEDLINE on STN

92355728. PubMed ID: 1379606. Direct sequencing of large **flavivirus** PCR products for analysis of genome variation and molecular epidemiological investigations. Lewis J G; **Chang G J**; Lanciotti R S; Trent D W. (Division of Vector-Borne Infectious Diseases, Centers for Disease Control, Fort Collins, CO 80522. ) Journal of virological methods, (1992 Jul) Vol. 38, No. 1, pp. 11-23. Journal code: 8005839. ISSN: 0166-0934. Pub. country: Netherlands. Language: English.

AB The polymerase chain reaction (PCR) was used to amplify viral cDNAs from selected regions of **dengue** genomic RNA by using appropriate 'consensus' primers. DNA amplicons containing the structural genes from all 4 **dengue** serotypes were prepared and directly sequenced using **dengue**-virus-specific primers. This method can characterize reliably **flavivirus** field isolates at the molecular level without extensive virus propagation and molecular cloning, and will be a valuable tool for molecular epidemiological studies.

L24 ANSWER 15 OF 16 MEDLINE on STN

92202358. PubMed ID: 1372617. Rapid detection and typing of **dengue** viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. Lanciotti R S; Calisher C H; Gubler D J; **Chang G J**; Vorndam A V. (Division of Vector-Borne Infectious Diseases, Centers for Disease Control, Fort Collins, Colorado 80522. ) Journal of clinical microbiology, (1992 Mar) Vol. 30, No. 3, pp. 545-51. Journal code: 7505564. ISSN: 0095-1137. Pub. country: United States. Language: English.

AB We report on the development and application of a rapid assay for detecting and typing **dengue** viruses. Oligonucleotide consensus primers were designed to anneal to any of the four **dengue** virus types and amplify a 511-bp product in a reverse transcriptase-polymerase chain reaction (PCR). First, we produced a cDNA copy of a portion of the viral genome in a reverse transcriptase reaction in the presence of primer D2 and then carried out a standard PCR (35 cycles of heat denaturation, annealing, and primer extension) with the addition of primer D1. The resulting double-stranded DNA product of the RT-PCR was typed by two methods: dot blot hybridization of the 511-bp amplified product to **dengue** virus type-specific probes or a second round of PCR amplification (nested PCR) with type-specific primers, yielding DNA products the unique sizes of which were diagnostic for each **dengue** virus serotype. The accumulated data demonstrated that **dengue** viruses can be accurately detected and typed from viremic human serum samples.

L24 ANSWER 16 OF 16 MEDLINE on STN

90320126. PubMed ID: 2371768. Nucleotide sequence of the virulent SA-14 strain of **Japanese encephalitis** virus and its attenuated vaccine

(Division of Vector-Borne Infections Diseases, Centers for Disease Control, Fort Collins, Colorado 80522. ) Virology, (1990 Aug) Vol. 177, No. 2, pp. 541-52. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB The attenuated SA-14-14-2 strain of Japanese encephalitis (**JE**) virus has been used to immunize people in the People's Republic of China. Oligonucleotide fingerprints of the parent SA-14 and vaccine strain indicate that multiple genetic changes occurred during attenuation of the virus. We have cloned and sequenced the genomes of both the virulent SA-14 and attenuated SA-14-14-2 viruses to define molecular differences in the genomes. Forty-five nucleotide differences, resulting in 15 amino acid substitutions, were found by comparing sequences of the SA-14 and SA-14-14-2 genomes. Transversion of U to A occurred at position 39 in the 5'-noncoding region of SA-14-14-2 and another SA-14 vaccine derivative SA-14-5-3. A single nucleotide change in the capsid gene of SA-14-14-2 altered a single amino acid which changed its predicted secondary structure. A silent nucleotide change was found in the prM gene sequence and the M-protein was unchanged. There are seven nucleotide differences, resulting in five amino acid changes, in the E glycoprotein sequence of the two viruses. Nine amino acid differences were found in the nonstructural proteins of SA-14 and SA-14-14-2: one in NS2A, two in NS2B, three in NS3, one in ns4a, and two in NS5. A single nucleotide change at position 10,428 in the 3'-noncoding region is vaccine virus-specific. The nucleotide and deduced amino acid sequences of the vaccine strain SA-14-14-2, the parent virus SA-14, and virulent strains JaOArS982 and Beijing-1 have been compared and are highly conserved.

=> e konishi e/au

E1	2	KONISHI DE TOFFOLI G/AU
E2	1	KONISHI DE TOFFOLI J/AU
E3	97 -->	KONISHI E/AU
E4	1	KONISHI E J/AU
E5	6	KONISHI EIICHI/AU
E6	17	KONISHI EIJI/AU
E7	2	KONISHI EISAKU/AU
E8	7	KONISHI EMIKO/AU
E9	176	KONISHI F/AU
E10	1	KONISHI FUMIKAZU/AU
E11	4	KONISHI FUMIKO/AU
E12	43	KONISHI FUMIO/AU

=> s e6

L25 17 "KONISHI EIJI"/AU

=> d l25,ti,1-3

L25 ANSWER 1 OF 17 MEDLINE on STN

TI Natural infection with Japanese encephalitis virus among inhabitants of Japan: a nationwide survey of antibodies against nonstructural 1 protein.

L25 ANSWER 2 OF 17 MEDLINE on STN

TI Mosquito cells infected with Japanese encephalitis virus release slowly-sedimenting hemagglutinin particles in association with intracellular formation of smooth membrane structures.

L25 ANSWER 3 OF 17 MEDLINE on STN

TI Dengue tetravalent DNA vaccine inducing neutralizing antibody and anamnestic responses to four serotypes in mice.

=> s e5

L26 6 "KONISHI EIICHI"/AU

=> d l26,ti,1-3

L26 ANSWER 1 OF 6 MEDLINE on STN

TI A novel serine protease highly expressed in the pancreas is expressed in various kinds of cancer cells.

L26 ANSWER 2 OF 6 MEDLINE on STN

TI Irritated seborrheic keratosis of the external ear canal.

L26 ANSWER 3 OF 6 MEDLINE on STN

TI Clear cell carcinoid tumor of the gallbladder. A case without von Hippel-Lindau disease.

=> d his

(FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006)

E CHANG G J J/IN  
 E CHANG GWONG J J/IN  
 L1 2 S E4  
 E KONISHI E/AU  
 E KONISHI E/IN  
 L2 24958 S CMV OR CMV-IE  
 L3 3918 S L2 AND KOZAK  
 L4 3136 S L3 AND TERMINATION  
 L5 0 S L4 AND (POLY W A)  
 L6 760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR  
 L7 33 S L6 AND CMV/CLM  
 L8 11 S L7 AND AY<1999  
 L9 498 S L6 AND PCDNA3?  
 L10 9 S L9 AND AY<1999  
 L11 3 S L10 NOT L8  
 E SCHMALJOHN C S/IN  
 L12 12 S E4  
 L13 4840 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELLOW FEVER  
 L14 161 S L13 AND (PRM? AND E)  
 L15 82 S L14 AND (SIGNAL SEQUENCE)  
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FILE 'WPIDS' ENTERED AT 22:19:11 ON 01 JUN 2006

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 L26 6 S E5

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L25 ANSWER 1 OF 17 MEDLINE on STN

2006171855. PubMed ID: 16460843. Natural infection with Japanese  
 encephalitis virus among inhabitants of Japan: a nationwide survey of  
 antibodies against nonstructural 1 protein. **Konishi Eiji**; Shoda Mizue;  
 Yamamoto Seigo; Arai Satoru; Tanaka-Taya Keiko; Okabe Nobuhiko.  
 (Department of Health Sciences, Kobe University School of Medicine, 7-10-2  
 Tomogaoka, Suma-ku, Kobe 654-0142, Japan.. ekon@ams.kobe-u.ac.jp) .  
 Vaccine, (2006 Apr 12) Vol. 24, No. 16, pp. 3054-6. Electronic  
 Publication: 2006-01-19. Journal code: 8406899. ISSN: 0264-410X. Pub.  
 country: Netherlands. Language: English.

AB In Japan, only a few cases of Japanese encephalitis presently occur  
 annually, raising arguments about the necessity of vaccination. Here,  
 using a novel immunoassay, we obtained natural infection rates for 2001 of  
 0.2-3.4% in eight selected prefectures across Japan. Our results provide  
 direct evidence of the risk of reemergence.

L25 ANSWER 2 OF 17 MEDLINE on STN

2006157796. PubMed ID: 16547419. Mosquito cells infected with Japanese  
 encephalitis virus release slowly-sedimenting hemagglutinin particles in  
 association with intracellular formation of smooth membrane structures.  
 Ishikawa Tomohiro; **Konishi Eiji**. (Department of Health Sciences, Kobe  
 University School of Medicine, Kobe, Hyogo 654-0142, Japan. ) Microbiology  
 and immunology, (2006) Vol. 50, No. 3, pp. 211-23. Journal code: 7703966.  
 ISSN: 0385-5600. Pub. country: Japan. Language: English.

AB Arthropod-borne flaviviruses can grow in both arthropod and mammalian  
 cells. Virion morphogenesis, though well studied in mammalian cells, is  
 still unclear in arthropod cells. Here, we compared a mosquito cell line  
 C6/36 and a mammalian cell line Vero in extracellular virus particles and  
 intracellular ultrastructures triggered by infection with Japanese  
 encephalitis virus (JEV). Sedimentation analyses of virion and  
 slowly-sedimenting hemagglutinin (SHA) particles released by infection  
 with the Nakayama strain revealed that C6/36 cells produced higher  
 envelope (E) antigen levels in the SHA than the virion fraction in  
 contrast to Vero cells that showed the opposite pattern. Specific  
 infectivities per ng of E were similar in both cells, whereas specific  
 hemagglutinating activities in the SHA fraction were lower in C6/36 than  
 Vero cells. The precursor membrane protein was less efficiently cleaved  
 to the membrane protein in SHA particles released from C6/36 than Vero  
 cells. Ultrastructural studies showed more remarkable production of  
 smooth membrane structures (SMSs) in C6/36 than in Vero cells. The

between Nakayama-infected C6/36 and Vero cells were consistently observed in 5 other strains (Beijing P1, Beijing P3, JaTH-160, KE-093 and JaGAR-01), except for KE-093-infected C6/36 cells which exhibited the Vero-type sedimentation profile under conditions of open cultivation. By electron microscopy, the production of SMSs from KE-093-infected C6/36 cells under open conditions was markedly less than that under closed conditions where the cells exhibited the C6/36-type sedimentation profile. Thus, intracellular SMS formations were associated with extracellular SHA production in JEV-infected mosquito cells.

L25 ANSWER 3 OF 17 MEDLINE on STN

2006104316. PubMed ID: 16316713. Dengue tetravalent DNA vaccine inducing neutralizing antibody and anamnestic responses to four serotypes in mice. **Konishi Eiji**; Kosugi Saori; Imoto Jun-ichi. (Department of Health Sciences, Kobe University School of Medicine, 7-10-2 Tomogaoka, Suma-ku, Kobe 654-0142, Japan.. ekon@ams.kobe-u.ac.jp) . Vaccine, (2006 Mar 15) Vol. 24, No. 12, pp. 2200-7. Electronic Publication: 2005-11-15. Journal code: 8406899. ISSN: 0264-410X. Pub. country: Netherlands. Language: English.

AB We developed a dengue tetravalent DNA vaccine consisting of plasmids expressing premembrane and envelope genes of each of four serotypes of dengue viruses. BALB/c mice immunized twice with the tetravalent vaccine at a dose of 100 microg (25 microg for each serotype) using a needle-free jet injector developed neutralizing antibodies against all serotypes. There was no interference among the four components included in this combination vaccine. Tetravalent vaccine-immunized mice showed anamnestic neutralizing antibody responses following challenge with each dengue serotype: responses to challenges from serotypes different to those used for neutralization tests were also induced.

L25 ANSWER 4 OF 17 MEDLINE on STN

2005697147. PubMed ID: 16129523. Analysis of yearly changes in levels of antibodies to Japanese encephalitis virus nonstructural 1 protein in racehorses in central Japan shows high levels of natural virus activity still exist. **Konishi Eiji**; Shoda Mizue; Kondo Takashi. (Department of Health Sciences, Kobe University School of Medicine, 7-10-2 Tomogaoka, Suma-ku, Kobe 654-0142, Japan.. ekon@ams.kobe-u.ac.jp) . Vaccine, (2006 Jan 23) Vol. 24, No. 4, pp. 516-24. Electronic Publication: 2005-08-11. Journal code: 8406899. ISSN: 0264-410X. Pub. country: Netherlands. Language: English.

AB Recent reductions in numbers of human and equine Japanese encephalitis (JE) cases in Japan have seen calls to end JE vaccination. Here, we analyzed yearly variations of natural JE virus activity, using sera collected serially in 1998-2003 from racehorses residing in Ibaraki and Shiga prefectures, both located in central Japan. A total of 208 sera from 24 individuals in Ibaraki and 259 from 27 in Shiga were examined for antibodies to JE virus nonstructural 1 (NS1) protein, a marker of natural infection. The natural infection rate in epizootic seasons, which was determined by a significant increase in NS1 antibody level, was 4.2-26.7% in Ibaraki and 0-41.7% in Shiga, indicating that high levels of JE virus activity still existed in central Japan.

L25 ANSWER 5 OF 17 MEDLINE on STN

2005676304. PubMed ID: 16363686. Clinical and epidemiological aspects of Japanese encephalitis. **Konishi Eiji**. (Department of Health Sciences, Kobe University School of Medicine. ) Nippon rinsho. Japanese journal of clinical medicine, (2005 Dec) Vol. 63, No. 12, pp. 2138-42. Ref: 12. Journal code: 0420546. ISSN: 0047-1852. Pub. country: Japan. Language: Japanese.

AB Japanese encephalitis has been well controlled in Japan. On the other hand, the Japanese encephalitis virus (JEV) transmission cycle still exists, as does the risk of exposure to JEV infection. Although *Culex tritaeniorhynchus* has been considered the major mosquito vector in Japan, recent surveys suggested *Culex pipiens* complex and *Aedes albopictus* as potential alternatives. Similarly, swine have reduced the role in amplification of JEV in paredomestic environments. Another surveys suggested attenuation of recent JEV strains and a shift in the major clinical manifestation caused by JEV infection from encephalitis to meningitis. This paper describes current situation of Japanese encephalitis in Japan from clinical and epidemiological aspects.

L25 ANSWER 6 OF 17 MEDLINE on STN

2005171219. PubMed ID: 15802965. Needle-free jet injection of a mixture of Japanese encephalitis DNA and protein vaccines: a strategy to effectively enhance immunogenicity of the DNA vaccine in a murine model. Imoto Jun-Ichi; **Konishi Eiji**. (Department of Health Sciences, Kobe University School of Medicine, 7-10-2 Tomogaoka, Suma-ku, Kobe 654-0142, Japan. ) Viral immunology, (2005) Vol. 18, No. 1, pp. 205-12. Journal code: 8801552. ISSN: 0882-8245. Pub. country: United States. Language: English.

AB Combined immunization with gene-based and protein-based vaccines can increase vaccine effectiveness. We previously demonstrated, using a murine model for Japanese encephalitis (JE), that simultaneous

protein vaccine consisting of subviral extracellular particles (EPs) by the subcutaneous route provided a synergistic increase in immunogenicities of these vaccines. Here, we investigated a novel immunization protocol consisting of a single inoculation with a mixture of DNA and protein vaccines using a needle-free jet injector. Immunization of ddY mice with 1 microg of pcJEME mixed with 1 microg of EPs or a 1/100 dose of commercial inactivated JE vaccine (JEVAX) induced neutralizing antibody titers of 1:40 to 1:80 (90% plaque reduction) 6 weeks after immunization, whereas immunization with DNA or protein alone only induced low titers ( $\leq 1:10$ ). Co-immunization with pcDNA3, a CpG-containing vector of the vaccine plasmid, increased immunogenicity of JEVAX to some extent. IgG1/IgG2a isotype profiles supported increased production of EPs in pcJEME-inoculated mice by needle-free injection and an adjuvant effect of the vector on immunogenicity of JEVAX.

L25 ANSWER 7 OF 17 MEDLINE on STN

2005100910. PubMed ID: 15731239. Nuclear localization of Japanese encephalitis virus core protein enhances viral replication. Mori Yoshio; Okabayashi Tamaki; Yamashita Tetsuo; Zhao Zijiang; Wakita Takaji; Yasui Kotaro; Hasebe Futoshi; Tadano Masayuki; **Konishi Eiji**; Moriishi Kohji; Matsuura Yoshiharu. (Research Center for Emerging Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. ) Journal of virology, (2005 Mar) Vol. 79, No. 6, pp. 3448-58. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB Japanese encephalitis virus (JEV) core protein was detected in both the nucleoli and cytoplasm of mammalian and insect cell lines infected with JEV or transfected with the expression plasmid of the core protein. Mutation analysis revealed that Gly(42) and Pro(43) in the core protein are essential for the nuclear and nucleolar localization. A mutant M4243 virus in which both Gly(42) and Pro(43) were replaced by Ala was recovered by plasmid-based reverse genetics. In C6/36 mosquito cells, the M4243 virus exhibited RNA replication and protein synthesis comparable to wild-type JEV, whereas propagation in Vero cells was impaired. The mutant core protein was detected in the cytoplasm but not in the nucleus of either C6/36 or Vero cell lines infected with the M4243 virus. The impaired propagation of M4243 in mammalian cells was recovered by the expression of wild-type core protein in trans but not by that of the mutant core protein. Although M4243 mutant virus exhibited a high level of neurovirulence comparable to wild-type JEV in spite of the approximately 100-fold-lower viral propagation after intracerebral inoculation to 3-week-old mice of strain Jc1:ICR, no virus was recovered from the brain after intraperitoneal inoculation of the mutant. These results indicate that nuclear localization of JEV core protein plays crucial roles not only in the replication in mammalian cells in vitro but also in the pathogenesis of encephalitis induced by JEV in vivo.

L25 ANSWER 8 OF 17 MEDLINE on STN

2004556484. PubMed ID: 15528700. Development and evaluation of an enzyme-linked immunosorbent assay for quantifying antibodies to Japanese encephalitis virus nonstructural 1 protein to detect subclinical infections in vaccinated horses. **Konishi Eiji**; Shoda Mizue; Ajiro Naoko; Kondo Takashi. (Department of Health Sciences, Kobe University School of Medicine, Suma-ku, Kobe 654-0142, Japan.. ekon@ams.kobe-u.ac.jp) . Journal of clinical microbiology, (2004 Nov) Vol. 42, No. 11, pp. 5087-93. Journal code: 7505564. ISSN: 0095-1137. Pub. country: United States. Language: English.

AB Antibodies to Japanese encephalitis virus (JEV) nonstructural 1 (NS1) protein constitute a marker of natural JEV infection among populations vaccinated with inactivated JE vaccine. In Japan, with few recent human JE cases, the natural infection rate is critical to evaluate the necessity of continuing JE vaccination. A sensitive immunochemical staining method for detecting NS1 antibodies in individuals naturally and subclinically infected with JEV was previously established. Here, an enzyme-linked immunosorbent assay (ELISA) to detect NS1 antibodies in equine sera was developed and evaluated as an alternative to immunostaining. By this method, NS1 antigens contained in culture fluids from cells stably transfected with the NS1 and NS2A genes were captured by a rabbit anti-NS1 polyclonal antibody. Three nanograms per well of NS1 antigen, corresponding to 1:2 to 1:8 dilutions of the culture fluid, was sufficient for testing. ELISA values were obtained by a single-serum dilution (1:100), which correlated with ELISA titers obtained by an endpoint method. Under a tentative cutoff value (0.122) statistically calculated from NS1 antibody levels of horses in an area where JEV is not endemic, a high level of qualitative agreement (85.3%) was obtained between the ELISA and immunostaining methods. A significant correlation coefficient (0.799;  $P < 0.001$ ) was also obtained between the two methods. Three experimentally infected horses seroconverted no later than 13 to 23 days postinfection, whereas 4 field horses infected during an epizootic remained positive for NS1 antibodies for at least 40 weeks. Our results indicate that the ELISA used here was sufficiently sensitive to detect subclinical infections in vaccinated equine populations.

L25 ANSWER 9 OF 17 MEDLINE on STN

2004114784. PubMed ID: 15003636. Prevalence of antibody to Japanese encephalitis virus nonstructural 1 protein among racehorses in Japan: indication of natural infection and need for continuous vaccination. **Konishi Eiichi**; Shoda Mizue; Kondo Takashi. (Department of Health Sciences, Kobe University School of Medicine, 7-10-2 Tomokaoka, Suma-ku, Kobe 654-0142, Japan.. ekon@ams.kobe-u.ac.jp) . Vaccine, (2004 Mar 12) Vol. 22, No. 9-10, pp. 1097-103. Journal code: 8406899. ISSN: 0264-410X. Pub. country: Netherlands. Language: English.

AB Japanese encephalitis virus (JEV) causes fatal diseases in equines as well as humans. In Japan, racehorses are vaccinated with inactivated JE vaccine every year and no equine JE cases have been reported since 1986. However, the current reduction in JEV activity in nature has raised an argument against the requirement of continuous vaccination. Here, we studied natural infection rates in racehorses to address the issue. To identify naturally-infected individuals from vaccinated populations, we used an immunostaining method for detecting antibodies to JEV nonstructural 1 (NS1) protein. A total of 779 horses in eight nationwide locations showed NS1 antibody prevalences ranging from 15 to 73%. NS1 antibody prevalences among 2-year-old individuals that had spent one epizootic season in a particular location, therefore representing annual infection rates, were 15-67%. Individuals aged  $\geq 3$  years showed higher NS1 antibody titers than the 2-year-olds, suggesting that NS1 antibody levels were boosted by repeated exposures to JEV antigen over  $\geq 2$  epizootic seasons. These results indicate that horses in Japan are exposed to natural JEV infections, confirming the need for continuous vaccination for protecting horses from JE.

L25 ANSWER 10 OF 17 MEDLINE on STN

2003447965. PubMed ID: 12922102. Evidence for antigen production in muscles by dengue and Japanese encephalitis DNA vaccines and a relation to their immunogenicity in mice. **Konishi Eiichi**; Terazawa Aya; Fujii Atsuko. (Department of Health Sciences, Kobe University School of Medicine, 7-10-2 Tomogaoka, Suma-ku, Kobe 654-0142, Japan.. ekon@ams.kobe-u.ac.jp) . Vaccine, (2003 Sep 8) Vol. 21, No. 25-26, pp. 3713-20. Journal code: 8406899. ISSN: 0264-410X. Pub. country: Netherlands. Language: English.

AB This study demonstrated viral antigen production in muscle tissues following inoculation with DNA vaccines and examined its relation to antibody induction in mice using the flavivirus system. To achieve detectable levels of antigen production, we used a needle-free jet injector and examined 10% homogenate of quadriceps muscle for viral antigens in a sandwich enzyme-linked immunosorbent assay. We compared DNA vaccines against dengue type 1 (designated pcD1ME), dengue type 2 (pcD2ME) and Japanese encephalitis (pcJEME). The amounts of viral envelope (E) antigen contained in muscle homogenate 1, 2, 3 and 4 days following inoculation with 50 microg of pcJEME were 1.1, 1.0, 0.3 and  $<0.1$  ng/ml, respectively. Muscles from pcD2ME- and pcD1ME-inoculated mice did not contain detectable levels of E antigen ( $<0.1$  ng/ml) during 4 days following inoculation. The E amounts released from Vero cells transfected with DNAs were in the order pcJEME>pcD2ME>pcD1ME. Levels of neutralizing antibody induced by two immunizations with 100 microg of each DNA vaccine using needle-free or normal needle/syringe injection systems also were in the order pcJEME>pcD2ME>pcD1ME, 2-11 weeks after the first immunization. However, the difference in antibody levels among three DNA vaccines 14-18 weeks after immunization was smaller than that in the early phase of immunization. These results provide fundamental information useful for developing combination DNA vaccines, such as a dengue tetravalent DNA vaccine, which require adjustment of immunogenicity of each component.

L25 ANSWER 11 OF 17 MEDLINE on STN

2003405831. PubMed ID: 12944676. High Toxoplasma antibody prevalence among inhabitants in Jakarta, Indonesia. Terazawa Aya; Muljono Rusli; Susanto Lisawati; Margono Sri S; **Konishi Eiichi**. (Department of Health Sciences, Kobe University School of Medicine, Kobe 654-0142, Japan. ) Japanese journal of infectious diseases, (2003 Jun) Vol. 56, No. 3, pp. 107-9. Journal code: 100893704. ISSN: 1344-6304. Pub. country: Japan. Language: English.

AB We examined IgG antibody to Toxoplasma in sera from 1,693 inhabitants aged 20-85 years in Jakarta. The seroprevalence rate was 70%, without any significant differences between males (71%) and females (69%). Some epidemiological factors contributing to the high prevalences of antibody to Toxoplasma in inhabitants of urban areas in Indonesia were discussed.

L25 ANSWER 12 OF 17 MEDLINE on STN

2003386617. PubMed ID: 12922097. Comparison of protective efficacies of plasmid DNAs encoding Japanese encephalitis virus proteins that induce neutralizing antibody or cytotoxic T lymphocytes in mice. **Konishi Eiichi**; Ajiro Naoko; Nukuzuma Chiyoko; Mason Peter W; Kurane Ichiro. (Department of Health Sciences, Kobe University School of Medicine, 7-10-2 Tomogaoka, Suma-ku, Kobe 654-0142, Japan.. ekon@ams.kobe-u.ac.jp) . Vaccine, (2003 Sep 8) Vol. 21, No. 25-26, pp. 3675-83. Journal code: 8406899. ISSN: 0264-410X. Pub. country: Netherlands. Language: English.

envelope (E) proteins of Japanese encephalitis (JE) virus (designated pcJEME) produce neutralizing antibodies and are protected from JE. To determine the role of the immune response to other viral proteins in protection, we constructed plasmid DNAs encoding other JE virus proteins and made a direct comparison among these plasmids using a mouse model. Cytotoxic T lymphocytes (CTLs) were induced by plasmids encoding capsid (C) or nonstructural proteins, NS1, NS2A, NS2B, NS3 or NS5. However, these plasmids provided only a partial protection against intraperitoneal challenge with a lethal dose of JE virus, whereas mice immunized with pcJEME were fully protected. In mice inoculated with CTL-inducing plasmids, high virus titers were detected in plasma immediately (1h) following challenge and in brain on day 4 post-challenge, but no virus infectivity was detected in plasma and brain of pcJEME-immunized mice during the 5 days following challenge. These results indicate that protection provided by the prM/E-encoding DNA consists of neutralizing antibody that prevents virus dissemination from the peripheral site to the brain, and that this antibody-mediated mechanism of protection is more efficient than the immunity induced by plasmids that generate CTL responses capable of killing JE virus-infected cells.

L25 ANSWER 13 OF 17 MEDLINE on STN

2003302384. PubMed ID: 12828869. Enhancing effect of vaxfectin on the ability of a Japanese encephalitis DNA vaccine to induce neutralizing antibody in mice. Nukuzuma Chiyoko; Ajiro Naoko; Wheeler Carl J; **Konishi Eiji**. (Department of Health Sciences, Kobe University School of Medicine, Kobe, Japan. ) *Viral immunology*, (2003) Vol. 16, No. 2, pp. 183-9. Journal code: 8801552. ISSN: 0882-8245. Pub. country: United States. Language: English.

AB Vaxfectin, a recently developed adjuvant, was evaluated for its enhancing effect on immunogenicity of a Japanese encephalitis (JE) DNA vaccine plasmid encoding the JE virus premembrane (prM) and envelope (E) genes (designated pcJEME), using BALB/c and ICR mice. Formulation of pcJEME with Vaxfectin provided > or =8-fold higher neutralizing antibody titers than those induced by pcJEME alone and reduced the amount of pcJEME to one-tenth to induce comparable levels of neutralizing antibody. Use of Vaxfectin did not alter a Th1 type IgG isotype immune response (IgG1 < IgG2a) induced by pcJEME in mice. These results indicate that Vaxfectin has an ability to enhance immunogenicity of pcJEME and is considered as a useful adjuvant for DNA vaccines in murine experimental models.

L25 ANSWER 14 OF 17 MEDLINE on STN

2003254780. PubMed ID: 12744864. Immunogenicity of a Japanese encephalitis DNA vaccine candidate in cynomolgus monkeys. Tanabayashi Kiyoshi; Mukai Ryoaburo; Yamada Akio; Takasaki Tomohiko; Kurane Ichiro; Yamaoka Masaoki; Terazawa Aya; **Konishi Eiji**. (Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases, Tsukuba 305-0843, Japan. ) *Vaccine*, (2003 Jun 2) Vol. 21, No. 19-20, pp. 2338-45. Journal code: 8406899. ISSN: 0264-410X. Pub. country: Netherlands. Language: English.

AB A Japanese encephalitis (JE) vaccine candidate encoding JE virus premembrane (prM) and envelope (E) genes, designated pNJEME, was evaluated for safety and immunogenicity in non-human primate, cynomolgus monkeys. pNJEME was constructed using a vector (pNGVL4a) designed to address some of the safety concerns of DNA vaccine. In two different experiments, two immunizations with 300 microg of pNJEME by intramuscular (i.m.) injection, and 3 microg of pNJEME using a gene gun, and three immunizations by i.m. injection with 500 microg of pNJEME were performed. All the three protocols induced low to high levels of neutralizing antibody, indicating an ability of pNJEME to induce neutralizing antibody in monkeys with a wide individual variation in response to pNJEME. In one experiment designed to compare the DNA vaccine with a commercial inactivated JE vaccine, three immunizations by i.m. inoculation with 300 microg of pNJEME or by gene gun administration with 3 microg of pNJEME induced similar levels of neutralizing antibody to those induced by three immunizations with a human dose of the inactivated vaccine in most monkeys. After intranasal challenge with the Beijing P3 or JaTH160 strain of JE virus, pNJEME-immunized monkeys showed anamnestic neutralizing antibody responses, indicating that pNJEME induced memory B cells which were responsive to infection with JE virus. No systemic and local reactions were observed in any monkeys after i.m. or gene gun inoculations with plasmid DNAs.

L25 ANSWER 15 OF 17 MEDLINE on STN

2003188853. PubMed ID: 12706666. Simultaneous immunization with DNA and protein vaccines against Japanese encephalitis or dengue synergistically increases their own abilities to induce neutralizing antibody in mice. **Konishi Eiji**; Terazawa Aya; Imoto Jun-ichi. (Department of Health Sciences, Kobe University School of Medicine, 7-10-2 Tomogaoka, Suma-ku, Kobe 654-0142, Japan.. ekon@ams.kobe-u.ac.jp) . *Vaccine*, (2003 May 16) Vol. 21, No. 17-18, pp. 1826-32. Journal code: 8406899. ISSN: 0264-410X. Pub. country: Netherlands. Language: English.

AB Gene-based and protein-based vaccines are two distinct types of vaccines. In this report, we examined if combined use of DNA and protein vaccines

murine models for Japanese encephalitis (JE) or dengue type 2 (DEN2). DNA vaccines for JE (pcJEME) or DEN2 (pcD2ME) were inoculated intramuscularly, and protein vaccines consisting of subviral extracellular particles (EPs) containing JE (JEEP) or DEN2 (D2EP) virus antigens were inoculated subcutaneously with Freund's adjuvant. Two immunizations of ICR mice with pcJEME and/or JEEP in the prime-boost protocol indicated that levels of neutralizing antibody induced by the pcJEME prime-JEEP boost vaccination were two to eight-fold higher than those induced by pcJEME alone, but were equivalent to those induced by JEEP alone and slightly higher than those induced by the JEEP prime-pcJEME boost regimen. On the other hand, simultaneous immunization of ICR mice with pcJEME and JEEP provided synergistically higher neutralizing antibody titers than those provided by immunization with either immunogen. Immunization with graded doses of pcJEME and JEEP confirmed the synergism. The synergistic increase in neutralizing antibody titer by simultaneous immunization with DNA and protein vaccines was also shown by immunization with pcD2ME and D2EP in ICR and ddY mice. Both IgG1 and IgG2a antibodies were induced by combined immunization with pcJEME and JEEP.

L25 ANSWER 16 OF 17 MEDLINE on STN

2002683597. PubMed ID: 12443667. Ratios of subclinical to clinical Japanese encephalitis (JE) virus infections in vaccinated populations: evaluation of an inactivated JE vaccine by comparing the ratios with those in unvaccinated populations. **Konishi Eiji**; Suzuki Tomoyuki. (Department of Health Sciences, Kobe University School of Medicine, 7-10-2 Tomogaoka, Suma-ku, Kobe 654-0142, Japan.. ekon@ams.kobe-u.ac.jp) . Vaccine, (2002 Nov 22) Vol. 21, No. 1-2, pp. 98-107. Journal code: 8406899. ISSN: 0264-410X. Pub. country: Netherlands. Language: English.

AB Japanese encephalitis (JE) virus is characterized as a virus that produces a large number of subclinical infections. In this report, we estimated a ratio of subclinical to clinical infections in vaccinated human populations who acquired natural infection with JE virus, and evaluated protective capacity of the currently approved inactivated JE vaccine by comparing the ratio with those reported for unvaccinated populations. We developed a sensitive immunostaining method for detecting nonstructural 1 (NS1) antibody to demonstrate JE virus infection in vaccinated individuals. Serum samples collected from human populations in western Japan showed NS1 antibody prevalences of approximately 10% in an urban area in 1981 and 1995 and 20% in a rural area from 1982 through 1983. Analysis of annual change in NS1 antibody titer using paired samples provided a mean duration of NS1 antibody responses of approximately 2 years, indicating that 5% of the urban population or 10% of the rural population acquired natural JE virus infection in 1 year. Based on the number of JE cases from 1982 through 1991 and the number of people acquiring natural infection, and on the assumption that annual infection rates obtained in the present study areas are representative of the infection rate in entire Japan except for non-endemic northern areas, the ratio of subclinical to clinical infections in vaccinated populations was estimated to be 2000000:1, which was 2000-80000 times higher than the ratio previously reported for unvaccinated populations.

L25 ANSWER 17 OF 17 MEDLINE on STN

2002076987. PubMed ID: 11803066. Dengue type 2 virus subviral extracellular particles produced by a stably transfected mammalian cell line and their evaluation for a subunit vaccine. **Konishi Eiji**; Fujii Atsuko. (Department of Health Sciences, Kobe University School of Medicine, 7-10-2 Tomogaoka, Suma-ku, 654-0142, Kobe, Japan.. ekon@ams.kobe-u.ac.jp) . Vaccine, (2002 Jan 15) Vol. 20, No. 7-8, pp. 1058-67. Journal code: 8406899. ISSN: 0264-410X. Pub. country: England: United Kingdom. Language: English.

AB A dengue subunit vaccine candidate was developed using a mammalian cell line continuously expressing subviral extracellular particles (EPs) of the New Guinea C (NGC) strain of dengue type 2 virus. The cell line, designated D cell line, maintained envelope (E) antigen production for at least 10 passages. The EPs contained an E protein biochemically and antigenically equivalent to authentic E produced by NGC-infected Vero cells. Two immunizations of BALB/c mice with purified EPs containing 100ng or 400ng of E induced moderate levels of neutralizing antibody and anamnestic neutralizing antibody responses were produced when these animals were challenged with dengue virus. The yield of E antigen from D cells was comparable to that from NGC-infected Vero cells. When D cells were transfected with the anti-apoptotic bcl-2 gene, the E antigen release increased approximately two-fold. These results indicate that D cell EPs are a promising non-infectious vaccine antigen for dengue.

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E10     2      KONISHI FUSAKO/AU
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E12     1      KONISHI G P/AU

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      50156 JAPANESE
      28653 ENCEPHALITIS
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      21763 YELLOW
      107428 FEVER
      3054 YELLOW FEVER
      (YELLOW(W)FEVER)
L28     16 L27 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELLOW
      FEVER)

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L2      24958 S CMV OR CMV-IE
L3      3918 S L2 AND KOZAK
L4      3136 S L3 AND TERMINATION
L5      0 S L4 AND (POLY W A)
L6      760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR
L7      33 S L6 AND CMV/CLM
L8      11 S L7 AND AY<1999
L9      498 S L6 AND PCDNA3?
L10     9 S L9 AND AY<1999
L11     3 S L10 NOT L8
      E SCHMALJOHN C S/IN
L12     12 S E4
L13     4840 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELLOW FEVER
L14     161 S L13 AND (PRM? AND E)
L15     82 S L14 AND (SIGNAL SEQUENCE)
L16     15 S L15 AND AY<1999

FILE 'WPIDS' ENTERED AT 22:19:11 ON 01 JUN 2006
      E CHANG G J/IN
      E CHANG G J J/IN
L17     121 S E2
L18     3 S L17 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS O
L19     853 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITITS VIRU OR YELLOW
L20     28 S L19 AND (PRM? AND E)
L21     4 S L20 AND CMV

FILE 'MEDLINE' ENTERED AT 22:28:38 ON 01 JUN 2006
      E CHANG G J J/AU
L22     50 S E2
L23     23 S L22 AND (FLAVIVIR? OR DENGUE OR YELLOW FEVER OR JAPANESE ENCE
L24     16 S L23 AND PY<2000
      E KONISHI E/AU
L25     17 S E6
L26     6 S E5
      E KONISHI EIJI/AU
L27     25 S E2-E4
L28     16 S L27 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELL

=> d l28,cbib,12-16

L28 ANSWER 12 OF 16 MEDLINE on STN
2003302384. PubMed ID: 12828869. Enhancing effect of vaxfectin on the
ability of a Japanese encephalitis DNA vaccine to induce neutralizing
antibody in mice. Nukuzuma Chiyoko; Ajiro Naoko; Wheeler Carl J; Konishi

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Kobe, Japan. ) Viral immunology, (2003) Vol. 16, No. 2, pp. 183-9.  
Journal code: 8801552. ISSN: 0882-8245. Pub. country: United States.  
Language: English.

L28 ANSWER 13 OF 16 MEDLINE on STN

2003254780. PubMed ID: 12744864. Immunogenicity of a **Japanese encephalitis** DNA vaccine candidate in cynomolgus monkeys. Tanabayashi Kiyoshi; Mukai Ryozauro; Yamada Akio; Takasaki Tomohiko; Kurane Ichiro; Yamaoka Masaoki; Terazawa Aya; **Konishi Eiji**. (Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases, Tsukuba 305-0843, Japan. ) Vaccine, (2003 Jun 2) Vol. 21, No. 19-20, pp. 2338-45. Journal code: 8406899. ISSN: 0264-410X. Pub. country: Netherlands. Language: English.

L28 ANSWER 14 OF 16 MEDLINE on STN

2003188853. PubMed ID: 12706666. Simultaneous immunization with DNA and protein vaccines against **Japanese encephalitis** or **dengue** synergistically increases their own abilities to induce neutralizing antibody in mice. **Konishi Eiji**; Terazawa Aya; Imoto Jun-ichi. (Department of Health Sciences, Kobe University School of Medicine, 7-10-2 Tomogaoka, Suma-ku, Kobe 654-0142, Japan.. ekon@ams.kobe-u.ac.jp) . Vaccine, (2003 May 16) Vol. 21, No. 17-18, pp. 1826-32. Journal code: 8406899. ISSN: 0264-410X. Pub. country: Netherlands. Language: English.

L28 ANSWER 15 OF 16 MEDLINE on STN

2002683597. PubMed ID: 12443667. Ratios of subclinical to clinical **Japanese encephalitis** (JE) virus infections in vaccinated populations: evaluation of an inactivated JE vaccine by comparing the ratios with those in unvaccinated populations. **Konishi Eiji**; Suzuki Tomoyuki. (Department of Health Sciences, Kobe University School of Medicine, 7-10-2 Tomogaoka, Suma-ku, Kobe 654-0142, Japan.. ekon@ams.kobe-u.ac.jp) . Vaccine, (2002 Nov 22) Vol. 21, No. 1-2, pp. 98-107. Journal code: 8406899. ISSN: 0264-410X. Pub. country: Netherlands. Language: English.

L28 ANSWER 16 OF 16 MEDLINE on STN

2002076987. PubMed ID: 11803066. **Dengue** type 2 virus subviral extracellular particles produced by a stably transfected mammalian cell line and their evaluation for a subunit vaccine. **Konishi Eiji**; Fujii Atsuko. (Department of Health Sciences, Kobe University School of Medicine, 7-10-2 Tomogaoka, Suma-ku, 654-0142, Kobe, Japan.. ekon@ams.kobe-u.ac.jp) . Vaccine, (2002 Jan 15) Vol. 20, No. 7-8, pp. 1058-67. Journal code: 8406899. ISSN: 0264-410X. Pub. country: England: United Kingdom. Language: English.

=> d his

(FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006)

FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

E CHANG G J J/IN  
E CHANG GWONG J J/IN  
L1 2 S E4  
E KONISHI E/AU  
E KONISHI E/IN  
L2 24958 S CMV OR CMV-IE  
L3 3918 S L2 AND KOZAK  
L4 3136 S L3 AND TERMINATION  
L5 0 S L4 AND (POLY W A)  
L6 760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR  
L7 33 S L6 AND CMV/CLM  
L8 11 S L7 AND AY<1999  
L9 498 S L6 AND PCDNA3?  
L10 9 S L9 AND AY<1999  
L11 3 S L10 NOT L8  
E SCHMALJOHN C S/IN  
L12 12 S E4  
L13 4840 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELLOW FEVER  
L14 161 S L13 AND (PRM? AND E)  
L15 82 S L14 AND (SIGNAL SEQUENCE)  
L16 15 S L15 AND AY<1999

FILE 'WPIDS' ENTERED AT 22:19:11 ON 01 JUN 2006

E CHANG G J J/IN  
E CHANG G J J/IN  
L17 121 S E2  
L18 3 S L17 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS O  
L19 853 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITITS VIRU OR YELLOW  
L20 28 S L19 AND (PRM? AND E)  
L21 4 S L20 AND CMV

FILE 'MEDLINE' ENTERED AT 22:28:38 ON 01 JUN 2006

L22 50 S E2  
 L23 23 S L22 AND (FLAVIVIR? OR DENGUE OR YELLOW FEVER OR JAPANESE ENCE  
 L24 16 S L23 AND PY<2000  
 E KONISHI E/AU  
 L25 17 S E6  
 L26 6 S E5  
 E KONISHI EIJI/AU  
 L27 25 S E2-E4  
 L28 16 S L27 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELL

=> s (JEV or japanese encephalitis virus)  
 431 JEV  
 50156 JAPANESE  
 28653 ENCEPHALITIS  
 413837 VIRUS  
 1089 JAPANESE ENCEPHALITIS VIRUS  
 (JAPANESE(W)ENCEPHALITIS(W)VIRUS)  
 L29 1144 (JEV OR JAPANESE ENCEPHALITIS VIRUS)

=> s l29 and (subviral particles)  
 502 SUBVIRAL  
 83653 PARTICLES  
 241 SUBVIRAL PARTICLES  
 (SUBVIRAL(W)PARTICLES)  
 L30 6 L29 AND (SUBVIRAL PARTICLES)

=> d l30,cbib,1-6

L30 ANSWER 1 OF 6 MEDLINE on STN  
 2005308978. PubMed ID: 15956595. Histidine at residue 99 and the  
 transmembrane region of the precursor membrane prM protein are important  
 for the prM-E heterodimeric complex formation of **Japanese encephalitis  
 virus**. Lin Ying-Ju; Wu Suh-Chin. (Institute of Biotechnology, Department  
 of Life Science, National Tsing-Hua University, Hsinchu 30013, Taiwan,  
 Republic of China. ) Journal of virology, (2005 Jul) Vol. 79, No. 13, pp.  
 8535-44. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United  
 States. Language: English.

L30 ANSWER 2 OF 6 MEDLINE on STN  
 2004221400. PubMed ID: 15119774. A novel principle of attenuation for the  
 development of new generation live flavivirus vaccines. Kofler R M; Heinz  
 F X; Mandl C W. (Institute of Virology, University of Vienna, Vienna,  
 Austria. ) Archives of virology. Supplementum, (2004) No. 18, pp. 191-200.  
 Ref: 24. Journal code: 9214275. ISSN: 0939-1983. Pub. country: Austria.  
 Language: English.

L30 ANSWER 3 OF 6 MEDLINE on STN  
 2003108910. PubMed ID: 12620809. Enhancing biosynthesis and secretion of  
 premembrane and envelope proteins by the chimeric plasmid of dengue virus  
 type 2 and **Japanese encephalitis virus**. Chang Gwong-Jen J; Hunt Ann  
 R; Holmes Derek A; Springfield Tracy; Chiueh Tzong-Shi; Roehrig John T;  
 Gubler Duane J. (Division of Vector-Borne Infectious Diseases, Centers for  
 Disease Control and Prevention, Public Health Service, U.S. Department of  
 Health and Human Service, Post Office Box 2087, Fort Collins, CO 80522,  
 USA.. gxc7@cdc.gov) . Virology, (2003 Feb 1) Vol. 306, No. 1, pp. 170-80.  
 Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States.  
 Language: English.

L30 ANSWER 4 OF 6 MEDLINE on STN  
 2001180918. PubMed ID: 11160724. Generation and characterization of a  
 mammalian cell line continuously expressing **Japanese encephalitis  
 virus subviral particles**. Konishi E; Fujii A; Mason P W. (Department  
 of Health Sciences, Kobe University School of Medicine, Kobe 654-0142,  
 Japan.. ekon@ams.kobe-u.ac.jp) . Journal of virology, (2001 Mar) Vol. 75,  
 No. 5, pp. 2204-12. Journal code: 0113724. ISSN: 0022-538X. Pub. country:  
 United States. Language: English.

L30 ANSWER 5 OF 6 MEDLINE on STN  
 95266302. PubMed ID: 7747465. Sindbis vectors suppress secretion of  
**subviral particles** of **Japanese encephalitis virus** from mammalian  
 cells infected with SIN-JEV recombinants. Pugachev K V; Mason P W; Frey  
 T K. (Department of Biology, Georgia State University, Atlanta 30303, USA.  
 ) Virology, (1995 May 10) Vol. 209, No. 1, pp. 155-66. Journal code:  
 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

L30 ANSWER 6 OF 6 MEDLINE on STN  
 92263775. PubMed ID: 1585642. Mice immunized with a subviral particle  
 containing the **Japanese encephalitis virus** prM/M and E proteins are  
 protected from lethal **JEV** infection. Konishi E; Pincus S; Paoletti E;  
 Shope R E; Burrage T; Mason P W. (Department of Epidemiology and Public  
 Health, Yale University School of Medicine, New Haven, Connecticut 06510.  
 ) Virology, (1992 Jun) Vol. 188, No. 2, pp. 714-20. Journal code:

=> d 130,cbib,ab,5,6

L30 ANSWER 5 OF 6 MEDLINE on STN

95266302. PubMed ID: 7747465. Sindbis vectors suppress secretion of **subviral particles of Japanese encephalitis virus** from mammalian cells infected with SIN-JEV recombinants. Pugachev K V; Mason P W; Frey T K. (Department of Biology, Georgia State University, Atlanta 30303, USA. ) Virology, (1995 May 10) Vol. 209, No. 1, pp. 155-66. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB Double-subgenomic Sindbis virus (dsSIN) recombinants that express cassettes encoding prM-E or a C-terminally truncated form of E of **Japanese encephalitis virus (JEV)** were constructed. The products were efficiently expressed in both mammalian and mosquito cell lines infected with the dsSIN recombinants. However, suppression of prM-E secretion from mammalian cells infected with dsSIN-prM-E recombinants was observed. This suppression was more pronounced late in infection (< 5% of total product was secreted during an 8-hr chase) than early in infection (15% secretion during a 6-hr chase). In comparison, a vaccinia virus-prM-E recombinant (vP829) described previously (E. Konishi et al. (1991) Virology 185, 401-410) was shown to secrete 35-50% of total product during a 6- to 8-hr chase both early and late in infection. In contrast, secretion of prM-E from dsSIN-prM-E-infected mosquito (C6/36) cells was found to be efficient (> 50% during an 8-hr chase). The prM-E secreted from both mammalian and mosquito cells was in the form of **subviral particles** as determined by velocity gradient centrifugation, sensitivity to nonionic detergent, and analysis of processing of N-linked glycans. The truncated E protein expressed by the dsSIN recombinants was secreted efficiently from both mammalian and mosquito cells. Coinfection experiments with the dsSIN-JEV recombinants + wild-type vaccinia virus and vP829 + SIN demonstrated that the reduced level of secretion of **subviral particles** exhibited by the dsSIN-JEV recombinants was due to an inhibitory effect of the dsSIN vectors. Furthermore, this inhibitory effect was accounted for by the SIN nonstructural proteins since SIN replicons that express prM-E cassette in place of the SIN structural protein open reading frame exhibited a low level of subviral particle secretion. No self-propagating infectious particles were produced in cells transfected with SIN replicons that encode the **JEV** prM-E cassette. The suppression of subviral particle secretion was apparently correlated with the inhibition of cell protein synthesis which is mediated in SIN-infected vertebrate cells by expression of the SIN nonstructural proteins.

L30 ANSWER 6 OF 6 MEDLINE on STN

92263775. PubMed ID: 1585642. Mice immunized with a subviral particle containing the **Japanese encephalitis virus** prM/M and E proteins are protected from lethal **JEV** infection. Konishi E; Pincus S; Paoletti E; Shope R E; Burrage T; Mason P W. (Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut 06510. ) Virology, (1992 Jun) Vol. 188, No. 2, pp. 714-20. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB Extracellular **subviral particles** produced by HeLa cells infected with a recombinant vaccinia virus encoding the prM and E genes of **Japanese encephalitis virus (JEV)** were purified and characterized. These particles contained the **JEV** prM/M and E proteins embedded in a lipid bilayer, and RNA was not detected in particles using the polymerase chain reaction and primers recognizing a part of the **JEV** E gene. The particles were uniformly spherical with a 20-nm diameter and had 5-nm projections on their surface. Mice that received a single inoculation of the purified extracellular particles emulsified with Freund's complete adjuvant were fully protected against  $4.9 \times 10^5$  LD50 of **JEV**. Comparison of the neutralizing and hemagglutination-inhibiting antibody titers and radioimmunoprecipitation data showed that immunization with the particles induced an immune response similar to that following inoculation with the recombinant vaccinia virus.

=> d

L30 ANSWER 1 OF 6 MEDLINE on STN

Full Text

AN 2005308978 MEDLINE

DN PubMed ID: 15956595

TI Histidine at residue 99 and the transmembrane region of the precursor membrane prM protein are important for the prM-E heterodimeric complex formation of **Japanese encephalitis virus**.

AU Lin Ying-Ju; Wu Suh-Chin

CS Institute of Biotechnology, Department of Life Science, National Tsing-Hua University, Hsinchu 30013, Taiwan, Republic of China.

SO Journal of virology, (2005 Jul) Vol. 79, No. 13, pp. 8535-44. Journal code: 0113724. ISSN: 0022-538X.

DT Journal; Article; (JOURNAL ARTICLE)  
LA English  
FS Priority Journals  
EM 200508  
ED Entered STN: 16 Jun 2005  
Last Updated on STN: 20 Aug 2005  
Entered Medline: 19 Aug 2005

=> d his

(FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006)

FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

E CHANG G J J/IN  
E CHANG GWONG J J/IN  
L1 2 S E4  
E KONISHI E/AU  
E KONISHI E/IN  
L2 24958 S CMV OR CMV-IE  
L3 3918 S L2 AND KOZAK  
L4 3136 S L3 AND TERMINATION  
L5 0 S L4 AND (POLY W A)  
L6 760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR  
L7 33 S L6 AND CMV/CLM  
L8 11 S L7 AND AY<1999  
L9 498 S L6 AND PCDNA3?  
L10 9 S L9 AND AY<1999  
L11 3 S L10 NOT L8  
E SCHMALJOHN C S/IN  
L12 12 S E4  
L13 4840 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELLOW FEVER  
L14 161 S L13 AND (PRM? AND E)  
L15 82 S L14 AND (SIGNAL SEQUENCE)  
L16 15 S L15 AND AY<1999

FILE 'WPIDS' ENTERED AT 22:19:11 ON 01 JUN 2006

E CHANG G J J/IN  
E CHANG G J J/IN  
L17 121 S E2  
L18 3 S L17 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS O  
L19 853 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITITS VIRU OR YELLOW  
L20 28 S L19 AND (PRM? AND E)  
L21 4 S L20 AND CMV

FILE 'MEDLINE' ENTERED AT 22:28:38 ON 01 JUN 2006

E CHANG G J J/AU  
L22 50 S E2  
L23 23 S L22 AND (FLAVIVIR? OR DENGUE OR YELLOW FEVER OR JAPANESE ENCE  
L24 16 S L23 AND PY<2000  
E KONISHI E/AU  
L25 17 S E6  
L26 6 S E5  
E KONISHI EIJI/AU  
L27 25 S E2-E4  
L28 16 S L27 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELL  
L29 1144 S (JEV OR JAPANESE ENCEPHALITIS VIRUS)  
L30 6 S L29 AND (SUBVIRAL PARTICLES)

=> d cost

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
CONNECT CHARGES	8.91	163.62
NETWORK CHARGES	1.62	6.48
DISPLAY CHARGES	9.74	158.02
	-----	-----
FULL ESTIMATED COST	20.27	328.12

IN FILE 'MEDLINE' AT 22:45:01 ON 01 JUN 2006

=> d his

(FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006)

FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

E CHANG G J J/IN  
E CHANG GWONG J J/IN  
L1 2 S E4  
E KONISHI E/AU  
E KONISHI E/IN  
L2 24958 S CMV OR CMV-IE

L4 3136 S L3 AND TERMINATION  
 L5 0 S L4 AND (POLY W A)  
 L6 760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR  
 L7 33 S L6 AND CMV/CLM  
 L8 11 S L7 AND AY<1999  
 L9 498 S L6 AND PCDNA3?  
 L10 9 S L9 AND AY<1999  
 L11 3 S L10 NOT L8  
 E SCHMALJOHN C S/IN  
 L12 12 S E4  
 L13 4840 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELLOW FEVER  
 L14 161 S L13 AND (PRM? AND E)  
 L15 82 S L14 AND (SIGNAL SEQUENCE)  
 L16 15 S L15 AND AY<1999

FILE 'WPIDS' ENTERED AT 22:19:11 ON 01 JUN 2006

E CHANG G J/IN  
 E CHANG G J J/IN

L17 121 S E2  
 L18 3 S L17 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS O  
 L19 853 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITITS VIRU OR YELLOW  
 L20 28 S L19 AND (PRM? AND E)  
 L21 4 S L20 AND CMV

FILE 'MEDLINE' ENTERED AT 22:28:38 ON 01 JUN 2006

E CHANG G J J/AU

L22 50 S E2  
 L23 23 S L22 AND (FLAVIVIR? OR DENGUE OR YELLOW FEVER OR JAPANESE ENCE  
 L24 16 S L23 AND PY<2000  
 E KONISHI E/AU  
 L25 17 S E6  
 L26 6 S E5  
 E KONISHI EIJI/AU  
 L27 25 S E2-E4  
 L28 16 S L27 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELL  
 L29 1144 S (JEV OR JAPANESE ENCEPHALITIS VIRUS)  
 L30 6 S L29 AND (SUBVIRAL PARTICLES)

=> s (flavivir? or yellow fever virus? or japanese encephalitis virus? or dengue)

3719 FLAVIVIR?  
 21763 YELLOW  
 107428 FEVER  
 443161 VIRUS?  
 938 YELLOW FEVER VIRUS?  
 (YELLOW(W)FEVER(W)VIRUS?)  
 50156 JAPANESE  
 28653 ENCEPHALITIS  
 443161 VIRUS?  
 1123 JAPANESE ENCEPHALITIS VIRUS?  
 (JAPANESE(W)ENCEPHALITIS(W)VIRUS?)  
 5142 DENGUE  
 L31 9634 (FLAVIVIR? OR YELLOW FEVER VIRUS? OR JAPANESE ENCEPHALITIS VIRUS  
 ? OR DENGUE)

=> s l31 and (PrM? or E)

1232 PRM?  
 715043 E  
 L32 1243 L31 AND (PRM? OR E)

=> s l32 and (PrM?)

1232 PRM?  
 L33 233 L32 AND (PRM?)

=> s l33 and (PrM and E)

633 PRM  
 715043 E  
 L34 194 L33 AND (PRM AND E)

=> s l34 and py<2000

12412454 PY<2000  
 (PY<20000000)  
 L35 88 L34 AND PY<2000

=> s l35 and (subviral particle? or virus-like particle?)

502 SUBVIRAL  
 121386 PARTICLE?  
 269 SUBVIRAL PARTICLE?  
 (SUBVIRAL(W)PARTICLE?)  
 413837 VIRUS  
 375647 LIKE  
 121386 PARTICLE?  
 2675 VIRUS-LIKE PARTICLE?

=> d 136,cbib,ab,1-5

L36 ANSWER 1 OF 5 MEDLINE on STN

96256767. PubMed ID: 8676481. Recombinant **subviral particles** from tick-borne encephalitis virus are fusogenic and provide a model system for studying **flavivirus** envelope glycoprotein functions. Schalich J; Allison S L; Stiasny K; Mandl C W; Kunz C; Heinz F X. (Institute of Virology, University of Vienna, Austria. ) Journal of virology, (1996 Jul) Vol. 70, No. 7, pp. 4549-57. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB Recombinant **subviral particles** (RSPs) obtained by coexpression of the envelope (**E**) and premembrane (**prM**) proteins of tick-borne encephalitis virus in COS cells (S. L. Allison, K. Stadler, C. W. Mandl, C. Kunz, and F. X. Heinz, J. Virol. 69:5816-5820, 1995) were extensively characterized and shown to be ordered structures containing envelope glycoproteins with structural and functional properties very similar to those in the virion envelope. The particles were spherical, with a diameter of about 30 nm and a buoyant density of 1.14 g/cm<sup>3</sup> in sucrose gradients. They contained mature **E** proteins with endoglycosidase H-resistant glycans as well as fully cleaved mature M proteins. Cleavage of **prM**, which requires an acidic pH in exocytic compartments, could be inhibited by treatment of transfected cells with ammonium chloride, implying a common maturation pathway for RSPs and virions. RSPs incorporated [14C]choline but not [3H]uridine, demonstrating that they contain lipid but probably lack nucleic acid. The envelope proteins of RSPs exhibited a native antigenic and oligomeric structure compared with virions, and incubation at an acidic pH (pH <6.5) induced identical conformational changes and structural rearrangements, including an irreversible quantitative conversion of dimers to trimers. The RSPs were also shown to be functionally active, inducing membrane fusion in a low-pH-dependent manner and demonstrating the same specific hemagglutination activity as whole virions. Tick-borne encephalitis virus RSPs thus represent an excellent model system for investigating the structural basis of viral envelope glycoprotein functions.

L36 ANSWER 2 OF 5 MEDLINE on STN

95363998. PubMed ID: 7637027. Synthesis and secretion of recombinant tick-borne encephalitis virus protein **E** in soluble and particulate form. Allison S L; Stadler K; Mandl C W; Kunz C; Heinz F X. (Institute of Virology, University of Vienna, Austria. ) Journal of virology, (1995 Sep) Vol. 69, No. 9, pp. 5816-20. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB A quantitative study was performed to investigate the requirements for secretion of recombinant soluble and particulate forms of the envelope glycoprotein **E** of tick-borne encephalitis (TBE) virus. Full-length **E** and a carboxy terminally truncated anchor-free form were expressed in COS cells in the presence and absence of **prM**, the precursor of the viral membrane protein M. Formation of a heteromeric complex with **prM** was found to be necessary for efficient secretion of both forms of **E**, whereas only low levels of anchor-free **E** were secreted in the absence of **prM**. The **prM**-mediated transport function could also be provided by coexpression of **prM** and **E** from separate constructs, but a **prM**-to-**E** ratio of greater than 1:1 did not further enhance secretion. Full-length **E** formed stable intracellular heterodimers with **prM** and was secreted as a **subviral particle**, whereas anchor-free **E** was not associated with particles and formed a less stable complex with **prM**, suggesting that **prM** interacts with both the ectodomain and anchor region of **E**.

L36 ANSWER 3 OF 5 MEDLINE on STN

95266302. PubMed ID: 7747465. Sindbis vectors suppress secretion of **subviral particles** of **Japanese encephalitis virus** from mammalian cells infected with SIN-JEV recombinants. Pugachev K V; Mason P W; Frey T K. (Department of Biology, Georgia State University, Atlanta 30303, USA. ) Virology, (1995 May 10) Vol. 209, No. 1, pp. 155-66. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB Double-subgenomic Sindbis virus (dsSIN) recombinants that express cassettes encoding **prM-E** or a C-terminally truncated form of **E** of **Japanese encephalitis virus** (JEV) were constructed. The products were efficiently expressed in both mammalian and mosquito cell lines infected with the dsSIN recombinants. However, suppression of **prM-E** secretion from mammalian cells infected with dsSIN-**prM-E** recombinants was observed. This suppression was more pronounced late in infection (< 5% of total product was secreted during an 8-hr chase) than early in infection (15% secretion during a 6-hr chase). In comparison, a vaccinia virus-**prM-E** recombinant (vP829) described previously (**E**. Konishi et al. (1991) Virology 185, 401-410) was shown to secrete 35-50% of total product during a 6- to 8-hr chase both early and late in infection. In contrast, secretion of **prM-E** from dsSIN-**prM-E**-infected mosquito (C6/36) cells was found to be efficient (> 50% during an 8-hr chase). The **prM-E** secreted from both mammalian and mosquito cells was in the form

centrifugation, sensitivity to nonionic detergent, and analysis of processing of N-linked glycans. The truncated **E** protein expressed by the dsSIN recombinants was secreted efficiently from both mammalian and mosquito cells. Coinfection experiments with the dsSIN-JEV recombinants + wild-type vaccinia virus and vP829 + SIN demonstrated that the reduced level of secretion of **subviral particles** exhibited by the dsSIN-JEV recombinants was due to an inhibitory effect of the dsSIN vectors. Furthermore, this inhibitory effect was accounted for by the SIN nonstructural proteins since SIN replicons that express **prM-E** cassette in place of the SIN structural protein open reading frame exhibited a low level of **subviral particle** secretion. No self-propagating infectious particles were produced in cells transfected with SIN replicons that encode the JEV **prM-E** cassette. The suppression of **subviral particle** secretion was apparently correlated with the inhibition of cell protein synthesis which is mediated in SIN-infected vertebrate cells by expression of the SIN nonstructural proteins.

L36 ANSWER 4 OF 5 MEDLINE on STN

93297134. PubMed ID: 8517028. Regulation of the late events in **flavivirus** protein processing and maturation. Yamshchikov V F; Compans R W. (Department of Microbiology, University of Alabama, Birmingham 35294. ) Virology, (1993 Jan) Vol. 192, No. 1, pp. 38-51. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB In order to determine the requirements for secretion of **flavivirus** structural proteins, we analyzed the expression of several West Nile **flavivirus** gene cassettes of different lengths in vaccinia virus expression systems. Expression of the longest cassette coding for the 5'-nontranslated region, proteins C through NS2B, and the protease domain of NS3, resulted in secretion of **prM-E** complexes and cleavage of **prM**. The presence and proper processing of the NS2A-NS2B-NS3 region appeared to be necessary for **prM-E** secretion. These proteins were released from cells mostly as membranous complexes which may represent empty viral envelopes. Cleavage of the membrane-associated intracellular form of protein C (C(i)) to produce the virion form (Ce) appeared to be critical for release of viral proteins. The presence and proper cleavage of the NS2A-NS2B-NS3 region were also found to be necessary for efficient C-**prM** cleavage by signalases. The NS2B-NS3 complex was implicated in cleavage of the intracellular form of protein C. Formation of a low level of **virus-like particles** was detected by electron microscopy. A model for virion formation, suggesting a critical role of the NS2B and NS3 proteins, is discussed.

L36 ANSWER 5 OF 5 MEDLINE on STN

92263775. PubMed ID: 1585642. Mice immunized with a **subviral particle** containing the **Japanese encephalitis virus prM/M** and **E** proteins are protected from lethal JEV infection. Konishi E; Pincus S; Paoletti E; Shope R E; Burrage T; Mason P W. (Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut 06510. ) Virology, (1992 Jun) Vol. 188, No. 2, pp. 714-20. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB Extracellular **subviral particles** produced by HeLa cells infected with a recombinant vaccinia virus encoding the **prM** and **E** genes of **Japanese encephalitis virus** (JEV) were purified and characterized. These particles contained the JEV **prM/M** and **E** proteins embedded in a lipid bilayer, and RNA was not detected in particles using the polymerase chain reaction and primers recognizing a part of the JEV **E** gene. The particles were uniformly spherical with a 20-nm diameter and had 5-nm projections on their surface. Mice that received a single inoculation of the purified extracellular particles emulsified with Freund's complete adjuvant were fully protected against  $4.9 \times 10(5)$  LD50 of JEV. Comparison of the neutralizing and hemagglutination-inhibiting antibody titers and radioimmunoprecipitation data showed that immunization with the particles induced an immune response similar to that following inoculation with the recombinant vaccinia virus.

=> d his

(FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006)

FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

	E CHANG G J J/IN
	E CHANG GWONG J J/IN
L1	2 S E4
	E KONISHI E/AU
	E KONISHI E/IN
L2	24958 S CMV OR CMV-IE
L3	3918 S L2 AND KOZAK
L4	3136 S L3 AND TERMINATION
L5	0 S L4 AND (POLY W A)
L6	760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR
L7	33 S L6 AND CMV/CLM



```

L9      498 S L6 AND PCDNA3?
L10     9 S L9 AND AY<1999
L11     3 S L10 NOT L8
        E SCHMALJOHN C S/IN
L12     12 S E4
L13     4840 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELLOW FEVER
L14     161 S L13 AND (PRM? AND E)
L15     82 S L14 AND (SIGNAL SEQUENCE)
L16     15 S L15 AND AY<1999

```

FILE 'WPIDS' ENTERED AT 22:19:11 ON 01 JUN 2006

E CHANG G J/IN  
E CHANG G J J/IN

```

L17     121 S E2
L18     3 S L17 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS O
L19     853 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITITS VIRU OR YELLOW
L20     28 S L19 AND (PRM? AND E)
L21     4 S L20 AND CMV

```

FILE 'MEDLINE' ENTERED AT 22:28:38 ON 01 JUN 2006

E CHANG G J J/AU

```

L22     50 S E2
L23     23 S L22 AND (FLAVIVIR? OR DENGUE OR YELLOW FEVER OR JAPANESE ENCE
L24     16 S L23 AND PY<2000
        E KONISHI E/AU
L25     17 S E6
L26     6 S E5
        E KONISHI EIJI/AU
L27     25 S E2-E4
L28     16 S L27 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELL
L29     1144 S (JEV OR JAPANESE ENCEPHALITIS VIRUS)
L30     6 S L29 AND (SUBVIRAL PARTICLES)
L31     9634 S (FLAVIVIR? OR YELLOW FEVER VIRUS? OR JAPANESE ENCEPHALITIS VI
L32     1243 S L31 AND (PRM? OR E)
L33     233 S L32 AND (PRM?)
L34     194 S L33 AND (PRM AND E)
L35     88 S L34 AND PY<2000
L36     5 S L35 AND (SUBVIRAL PARTICLE? OR VIRUS-LIKE PARTICLE?)

```

=> s l35 not l36

L37 83 L35 NOT L36

=> d l37,cbib,ab,70-83)

'70-83}' IS NOT A VALID FORMAT FOR FILE 'MEDLINE'

The following are valid formats:

The default display format is BIB.

```

ABS ---- AB
ALL ---- AN, DN, TI, AU, CS, NC, SO, CM, CY, DT, LA, FS, OS, EM,
        ED, AB, ST, CT, NA, RN, CN, GEN
BIB ---- AN, DN, TI, AU, CS, NC, SO, CY, DT, LA, FS, OS, EM, ED
CBIB --- AN, DN, TI, AU, CS, NC, SO, CY, DT, LA, FS, OS, EM, ED
DALL --- ALL, delimited for post processing
IABS --- ABS, with a text label
IALL --- ALL, indented with text labels
IBIB --- BIB, indented with text labels
IND ---- ST, CT, NA, RN, CN, GEN
SAM ---- TI, ST, CT, NA, RN, CN, GEN
TRI ---- TI, ST, CT, NA, RN, CN, GEN
TRIAL -- TI, ST, CT, NA, RN, CN, GEN
HIT ---- All fields containing hit terms
HITIND - IND
KWIC --- All hit terms plus 20 words on either side
OCC ---- List of display fields containing hit terms

```

Hit terms will be highlighted in all available fields except CM and PY.

To display a particular field or fields, enter the display field codes. For a list of display field codes, enter 'HELP DFIELDS' at an arrow prompt (=>). Examples of formats include: 'BIB'; 'AB'; 'SO,ST'. You may specify the format fields in any order, and the information will be displayed in the same order as the format specification.

The same formats (except for HIT, HITIND, KWIC, and OCC) may be used with the DISPLAY ACC command to display the record for a specified Accession Number.

ENTER DISPLAY FORMAT (BIB):d his

'D' IS NOT A VALID FORMAT FOR FILE 'MEDLINE'

The following are valid formats:

The default display format is BIB.

ABS ---- AB  
ALL ---- AN, DN, TI, AU, CS, NC, SO, CM, CY, DT, LA, FS, OS, EM,  
ED, AB, ST, CT, NA, RN, CN, GEN  
BIB ---- AN, DN, TI, AU, CS, NC, SO, CY, DT, LA, FS, OS, EM, ED  
CBIB --- AN, DN, TI, AU, CS, NC, SO, CY, DT, LA, FS, OS, EM, ED  
DALL --- ALL, delimited for post processing  
IABS --- ABS, with a text label  
IALL --- ALL, indented with text labels  
IBIB --- BIB, indented with text labels  
IND ---- ST, CT, NA, RN, CN, GEN  
SAM ---- TI, ST, CT, NA, RN, CN, GEN  
TRI ---- TI, ST, CT, NA, RN, CN, GEN  
TRIAL -- TI, ST, CT, NA, RN, CN, GEN  
HIT ---- All fields containing hit terms  
HITIND - IND  
KWIC --- All hit terms plus 20 words on either side  
OCC ---- List of display fields containing hit terms

Hit terms will be highlighted in all available fields except CM and PY.

To display a particular field or fields, enter the display field codes. For a list of display field codes, enter 'HELP DFIELDS' at an arrow prompt (=>). Examples of formats include: 'BIB'; 'AB'; 'SO,ST'. You may specify the format fields in any order, and the information will be displayed in the same order as the format specification.

The same formats (except for HIT, HITIND, KWIC, and OCC) may be used with the DISPLAY ACC command to display the record for a specified Accession Number.

ENTER DISPLAY FORMAT (BIB):ti

L37 ANSWER 1 OF 83 MEDLINE on STN  
TI Antibody responses of **dengue** fever patients to **dengue** 2 (New Guinea C strain) viral proteins.

=> d his

(FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006)

FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

E CHANG G J J/IN  
E CHANG GWONG J J/IN  
L1 2 S E4  
E KONISHI E/AU  
E KONISHI E/IN  
L2 24958 S CMV OR CMV-IE  
L3 3918 S L2 AND KOZAK  
L4 3136 S L3 AND TERMINATION  
L5 0 S L4 AND (POLY W A)  
L6 760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR  
L7 33 S L6 AND CMV/CLM  
L8 11 S L7 AND AY<1999  
L9 498 S L6 AND PCDNA3?  
L10 9 S L9 AND AY<1999  
L11 3 S L10 NOT L8  
E SCHMALJOHN C S/IN  
L12 12 S E4  
L13 4840 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELLOW FEVER  
L14 161 S L13 AND (PRM? AND E)  
L15 82 S L14 AND (SIGNAL SEQUENCE)  
L16 15 S L15 AND AY<1999

FILE 'WPIDS' ENTERED AT 22:19:11 ON 01 JUN 2006

E CHANG G J J/IN  
E CHANG G J J/IN  
L17 121 S E2  
L18 3 S L17 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS O  
L19 853 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITITS VIRU OR YELLOW  
L20 28 S L19 AND (PRM? AND E)  
L21 4 S L20 AND CMV

FILE 'MEDLINE' ENTERED AT 22:28:38 ON 01 JUN 2006

E CHANG G J J/AU  
L22 50 S E2  
L23 23 S L22 AND (FLAVIVIR? OR DENGUE OR YELLOW FEVER OR JAPANESE ENCE

L25 E KONISHI E/AU  
 L26 17 S E6  
 6 S E5  
 E KONISHI EIJI/AU  
 L27 25 S E2-E4  
 L28 16 S L27 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELL  
 L29 1144 S (JEV OR JAPANESE ENCEPHALITIS VIRUS)  
 L30 6 S L29 AND (SUBVIRAL PARTICLES)  
 L31 9634 S (FLAVIVIR? OR YELLOW FEVER VIRUS? OR JAPANESE ENCEPHALITIS VI  
 L32 1243 S L31 AND (PRM? OR E)  
 L33 233 S L32 AND (PRM?)  
 L34 194 S L33 AND (PRM AND E)  
 L35 88 S L34 AND PY<2000  
 L36 5 S L35 AND (SUBVIRAL PARTICLE? OR VIRUS-LIKE PARTICLE?)  
 L37 83 S L35 NOT L36

=> d 137,cbib,ab,kwic,70-83

L37 ANSWER 70 OF 83 MEDLINE on STN

91083519. PubMed ID: 2260923. Expression of the structural proteins of  
**dengue 2 virus** and **yellow fever virus** by recombinant vaccinia  
 viruses. Hahn Y S; Lenches E M; Galler R; Rice C M; Dalrymple J; Strauss J  
 H. (Division of Biology, California Institute of Technology, Pasadena. )  
 Archives of virology, (1990) Vol. 115, No. 3-4, pp. 251-65. Journal  
 code: 7506870. ISSN: 0304-8608. Pub. country: Austria. Language: English.

AB Vaccinia virus recombinants were constructed which contained cDNA  
 sequences encoding the structural region of **dengue 2 virus** (PR159/S1  
 strain) or **yellow fever virus** (17D strain). The **flavivirus** cDNA  
 sequences were expressed under the control of the vaccinia 7.5k early/late  
 promotor. Cultured cells infected with these recombinants expressed  
 immunologically reactive **flavivirus** structural proteins, precursor **prM**  
 and **E**. These proteins appeared to be cleaved and glycosylated properly  
 since they comigrated with the authentic proteins from **dengue 2 virus**-  
 and **yellow fever virus**-infected cells. Mice immunized with the  
**dengue/vaccinia** recombinant showed a **dengue**-specific immune response  
 that included low levels of neutralizing antibodies. Immunization of mice  
 with the yellow fever/vaccinia recombinant was less effective at inducing  
 an immune response to **yellow fever virus** and in only some of the  
 mice were low titers of neutralizing antibodies produced.

TI Expression of the structural proteins of **dengue 2 virus** and **yellow  
 fever virus** by recombinant vaccinia viruses.

SO Archives of virology, (1990) Vol. 115, No. 3-4, pp. 251-65.  
 Journal code: 7506870. ISSN: 0304-8608.

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 and **yellow fever virus**-infected cells. Mice immunized with the  
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 that included low levels of neutralizing antibodies. Immunization of mice  
 with the yellow fever/vaccinia recombinant was less effective at inducing  
 an immune response to **yellow fever virus** and in only some of the  
 mice were low titers of neutralizing antibodies produced.

CT Check Tags: Female; Male

Amino Acid Sequence

Animals

Antibodies, Viral: BI, biosynthesis

Antibodies, Viral: IM, immunology

\*Dengue Virus: GE, genetics

Dengue Virus: IM, immunology

Immunization

Mice

Mice, Inbred C57BL

Molecular Sequence Data

Neutralization Tests

Plasmids

Protein Precursors: BI, biosynthesis

Protein. . . Viral Envelope Proteins: IM, immunology

\*Viral Structural Proteins: BI, biosynthesis

Viral Structural Proteins: GE, genetics

Viral Structural Proteins: IM, immunology

\*Yellow fever virus: GE, genetics

Yellow fever virus: IM, immunology

CN 0 (Antibodies, Viral); 0 (E-glycoprotein, Dengue virus type 2); 0  
 (Protein Precursors); 0 (Viral Envelope Proteins); 0 (Viral Structural  
 Proteins)

L37 ANSWER 71 OF 83 MEDLINE on STN

recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. Mason P W; Pincus S; Fournier M J; Mason T L; Shope R E; Paoletti E. (Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut 06510. ) Virology, (1991 Jan) Vol. 180, No. 1, pp. 294-305. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB Four recombinant vaccinia viruses were engineered for expression of different portions of the **Japanese encephalitis virus** (JEV) open reading frame. All four recombinant vaccinia viruses contained the NS1 and NS2A genes, and each of these viruses specified the synthesis, glycosylation, and secretion of the nonstructural glycoprotein (NS1). All four recombinants also contained the **E** gene, and each virus correctly directed the synthesis and glycosylation of the envelope glycoprotein (**E**). Interestingly, two of these viruses (vP555 and vP650), which expressed the **prM** gene in addition to **E** and NS1, produced an extracellular hemagglutinin containing M and **E** that migrated in sucrose gradients similarly to the slowly-sedimenting hemagglutinin found in the culture fluid of JEV-infected cells. Immunization of 3-week-old mice with the recombinant viruses vP555 and vP658 resulted in immune responses to NS1, whereas only the virus that directed the synthesis of extracellular forms of **E** (vP555) induced an immune response to **E**. Both viruses provided protection against lethal challenge with JEV. Animals given two inoculations with vP555 were fully protected from greater than 10,000 LD50 of JEV. This high level of protection was correlated with the production of high titers of neutralizing and hemagglutination-inhibiting antibodies.

TI **Japanese encephalitis virus**-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection.

SO Virology, (1991 Jan) Vol. 180, No. 1, pp. 294-305.  
Journal code: 0110674. ISSN: 0042-6822.

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L37 ANSWER 72 OF 83 MEDLINE on STN

90320126. PubMed ID: 2371768. Nucleotide sequence of the virulent SA-14 strain of **Japanese encephalitis virus** and its attenuated vaccine derivative, SA-14-14-2. Nitayaphan S; Grant J A; Chang G J; Trent D W. (Division of Vector-Borne Infections Diseases, Centers for Disease Control, Fort Collins, Colorado 80522. ) Virology, (1990 Aug) Vol. 177, No. 2, pp. 541-52. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB The attenuated SA-14-14-2 strain of Japanese encephalitis (JE) virus has been used to immunize people in the People's Republic of China. Oligonucleotide fingerprints of the parent SA-14 and vaccine strain indicate that multiple genetic changes occurred during attenuation of the virus. We have cloned and sequenced the genomes of both the virulent SA-14 and attenuated SA-14-14-2 viruses to define molecular differences in the genomes. Forty-five nucleotide differences, resulting in 15 amino acid substitutions, were found by comparing sequences of the SA-14 and SA-14-14-2 genomes. Transversion of U to A occurred at position 39 in the 5'-noncoding region of SA-14-14-2 and another SA-14 vaccine derivative SA-14-5-3. A single nucleotide change in the capsid gene of SA-14-14-2 altered a single amino acid which changed its predicted secondary structure. A silent nucleotide change was found in the **prM** gene sequence and the M-protein was unchanged. There are seven nucleotide differences, resulting in five amino acid changes, in the **E** glycoprotein sequence of the two viruses. Nine amino acid differences were found in the nonstructural proteins of SA-14 and SA-14-14-2: one in NS2A, two in NS2B, three in NS3, one in ns4a, and two in NS5. A single nucleotide change at position 10,428 in the 3'-noncoding region is vaccine virus-specific. The nucleotide and deduced amino acid sequences of the vaccine strain SA-14-14-2, the parent virus SA-14, and virulent strains JaOArS982 and Beijing-1 have been compared and are highly conserved.

TI Nucleotide sequence of the virulent SA-14 strain of **Japanese encephalitis virus** and its attenuated vaccine derivative, SA-14-14-2.

SO Virology, (1990 Aug) Vol. 177, No. 2, pp. 541-52.

AB . . . SA-14-14-2 altered a single amino acid which changed its predicted secondary structure. A silent nucleotide change was found in the **prM** gene sequence and the M-protein was unchanged. There are seven nucleotide differences, resulting in five amino acid changes, in the **E** glycoprotein sequence of the two viruses. Nine amino acid differences were found in the nonstructural proteins of SA-14 and SA-14-14-2: . . .

L37 ANSWER 73 OF 83 MEDLINE on STN  
90281579. PubMed ID: 2353452. Production of **yellow fever virus** proteins in infected cells: identification of discrete polyprotein species and analysis of cleavage kinetics using region-specific polyclonal antisera. Chambers T J; McCourt D W; Rice C M. (Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110-1093. ) Virology, (1990 Jul) Vol. 177, No. 1, pp. 159-74. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB **Flavivirus** proteins are produced by translation of a single long open reading frame and a complex series of cotranslational and post-translational proteolytic cleavages. To study these processing events in **yellow fever virus** (YF)-infected cells, polyclonal antisera recognizing C, **prM**, **E**, NS1, NS2B, NS3, NS4B, and NS5 were generated using peptide and fusion protein immunogens. Evidence suggests that production of the structural protein precursors involves rapid cotranslational processing consistent with signalase cleavages. The synthesis of the NS1 glycoprotein involves cleavage of polyprotein precursors (t1/2 approximately 10 minutes) which probably contain portions of the NS2A gene product. Endoglycosidase F treatment or labeling in the presence of tunicamycin suggests that YF **prM** and NS1 each have two N-linked oligosaccharides. NS2B is produced without any identifiable precursors or associated polyprotein species. Processing of the NS3-4-5 region is complex and occurs rapidly. A series of polyproteins can be detected whose molecular weights correlate with the cleavage sites defined by available N-terminal amino acid sequence data. However, convincing precursor-product relationships between these polyproteins and the mature NS3 and NS5 proteins could not be demonstrated. In contrast, NS4B appears to be produced by cleavage of a discrete precursor believed to be NS4AB. N-terminal sequence data for the putative NS4AB product has tentatively defined the NS3-4A cleavage site. A scheme for in vivo processing of the YF polyprotein is presented and discussed.

TI Production of **yellow fever virus** proteins in infected cells: identification of discrete polyprotein species and analysis of cleavage kinetics using region-specific polyclonal antisera.

SO Virology, (1990 Jul) Vol. 177, No. 1, pp. 159-74. Journal code: 0110674. ISSN: 0042-6822.

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CT . . . U.S. Gov't, Non-P.H.S.  
Research Support, U.S. Gov't, P.H.S.  
Restriction Mapping  
Sulfur Radioisotopes  
Viral Proteins: BI, biosynthesis  
\*Viral Proteins: GE, genetics  
\*Yellow fever virus: GE, genetics  
Yellow fever virus: ME, metabolism

L37 ANSWER 74 OF 83 MEDLINE on STN  
89382762. PubMed ID: 2674479. Processing of **yellow fever virus** polyprotein: role of cellular proteases in maturation of the structural proteins. Ruiz-Linares A; Cahour A; Despres P; Girard M; Bouloy M. (Centre National de la Recherche Scientifique, UA 545, Institut Pasteur, Paris, France. ) Journal of virology, (1989 Oct) Vol. 63, No. 10, pp. 4199-209. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB The **yellow fever virus** (YFV) cDNA segment coding for the part of the precursor polyprotein generating the structural proteins C (capsid), **prM** (precursor to the membrane protein M), and **E** (envelope) was expressed in vitro by using the T7 promoter-polymerase transcription system coupled to translation in rabbit reticulocyte lysates. A polypeptide of the expected molecular weight was observed to accumulate in the assay and was processed into proteins C, **prM**, and **E** only when dog pancreas microsomal membranes were added to the translation system. Proteins **prM** and **E** were translocated inside the endoplasmic reticulum, where **prM** underwent glycosylation. Regions essential for translocation of these proteins were

proteins C and **prM**, respectively. Translocation of protein **prM** appeared to be less efficient than that of protein **E**. Maturation of these proteins followed different kinetics, indicating that the **prM** signal is probably cleaved off more slowly. A polypeptide composed of proteins C and **prM**, similar to the NVx polypeptide described in **yellow fever virus**-infected cells, was also produced in the in vitro system in the presence of membranes. No mature protein M was detected, suggesting that the cleavage of **prM** to M is a late processing event mediated by a protease different from endoplasmic reticulum signalases.

TI Processing of **yellow fever virus** polyprotein: role of cellular proteases in maturation of the structural proteins.

SO Journal of virology, (1989 Oct) Vol. 63, No. 10, pp. 4199-209.  
Journal code: 0113724. ISSN: 0022-538X.

AB The **yellow fever virus** (YFV) cDNA segment coding for the part of the precursor polyprotein generating the structural proteins C (capsid), **prM** (precursor to the membrane protein M), and **E** (envelope) was expressed in vitro by using the T7 promoter-polymerase transcription system coupled to translation in rabbit reticulocyte lysates. A polypeptide of the expected molecular weight was observed to accumulate in the assay and was processed into proteins C, **prM**, and **E** only when dog pancreas microsomal membranes were added to the translation system. Proteins **prM** and **E** were translocated inside the endoplasmic reticulum, where **prM** underwent glycosylation. Regions essential for translocation of these proteins were localized to the 18- and 15-amino-acid C-terminal hydrophobic regions of proteins C and **prM**, respectively. Translocation of protein **prM** appeared to be less efficient than that of protein **E**. Maturation of these proteins followed different kinetics, indicating that the **prM** signal is probably cleaved off more slowly. A polypeptide composed of proteins C and **prM**, similar to the NVx polypeptide described in **yellow fever virus**-infected cells, was also produced in the in vitro system in the presence of membranes. No mature protein M was detected, suggesting that the cleavage of **prM** to M is a late processing event mediated by a protease different from endoplasmic reticulum signalases.

CT . . . Research Support, Non-U.S. Gov't  
Viral Envelope Proteins: AN, analysis  
Viral Proteins: GE, genetics  
\*Viral Proteins: ME, metabolism  
Viral Structural Proteins  
\***Yellow fever virus: ME, metabolism**

L37 ANSWER 75 OF 83 MEDLINE on STN

89311624. PubMed ID: 2501515. In vitro processing of **dengue virus** structural proteins: cleavage of the pre-membrane protein. Markoff L. (Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892. ) Journal of virology, (1989 Aug) Vol. 63, No. 8, pp. 3345-52. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB Processing of **dengue virus** structural proteins was assessed in vitro. RNA transcripts for cell-free translation were prepared from cloned DNA (**dengue virus** type 4, strain 814669 genome) encoding capsid, pre-membrane (**prM**), and the first 23 amino acids of envelope (**E**). Processing of a 33-kilodalton precursor polypeptide encoded by wild-type RNA transcripts occurred only in the presence of added microsomal membranes. Under these conditions, cleavage at the capsid-**prM** and **prM-E** sites and glycosylation of **prM** occurred in association with translocation. Amino acid sequence analysis confirmed that translation initiated at the predicted N terminus of the capsid and that capsid-**prM** cleavage occurred at the predicted site for the action of signal peptidase following a candidate signal sequence (hydrophobic residues 100 to 113) in the **dengue virus** precursor. Mutations were introduced into the **dengue virus** DNA template by site-directed mutagenesis, altering nucleotide sequences encoding the capsid and the candidate signal for **prM**. The phenotypes of the mutants were deduced by analysis of the products of cell-free translation of the respective RNA transcripts. The resulting observations confirmed that cleavage at the capsid-**prM** and **prM-E** sites is effected entirely by signal peptidase and that the candidate signal is required for translocation.

TI In vitro processing of **dengue virus** structural proteins: cleavage of the pre-membrane protein.

SO Journal of virology, (1989 Aug) Vol. 63, No. 8, pp. 3345-52.  
Journal code: 0113724. ISSN: 0022-538X.

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CT Amino Acid Sequence  
Capsid: GE, genetics  
DNA, Viral: GE, genetics  
\***Dengue Virus: GE, genetics**  
**Dengue Virus: ME, metabolism**  
Glycoproteins: ME, metabolism  
Glycoside Hydrolases: ME, metabolism  
Mannosyl-Glycoprotein Endo-beta-N-Acetylglucosaminidase  
Mutation  
Precipitin Tests  
Protein Biosynthesis  
Protein Precursors: . . .

L37 ANSWER 76 OF 83 MEDLINE on STN

89299482. PubMed ID: 2741348. Definition of the carboxy termini of the three glycoproteins specified by **dengue** virus type 2. Wright P J; Cauchi M R; Ng M L. (Department of Microbiology, Monash University, Clayton, Victoria, Australia. ) Virology, (1989 Jul) Vol. 171, No. 1, pp. 61-7. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB The carboxy termini of the three glycoproteins (**prM**, **E**, and NS1) specified by **dengue** virus type 2 (DEN-2) were determined. The glycoproteins were radiolabeled with selected amino acids chosen following analysis of the deduced amino acid sequence of the polyprotein and then digested with carboxypeptidase A. The pattern of release of radioactive amino acids enabled definition of the carboxy termini. In addition, the amino terminus of NS2A was determined by Edman degradation of the radiolabeled protein. The results showed that no amino acids were lost at the carboxy termini of **prM**, **E**, and NS1 during their cleavage from the DEN-2 polyprotein. For each glycoprotein, the carboxy terminal amino acid immediately preceded the amino terminal acid of the following polypeptide.

TI Definition of the carboxy termini of the three glycoproteins specified by **dengue** virus type 2.

SO Virology, (1989 Jul) Vol. 171, No. 1, pp. 61-7.

Journal code: 0110674. ISSN: 0042-6822.

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CT Amino Acid Sequence  
Carboxypeptidases  
Carboxypeptidases A  
\***Dengue Virus: AN, analysis**  
\*Glycoproteins: AN, analysis  
Molecular Sequence Data  
Protein Precursors: AN, analysis  
Research Support, Non-U.S. Gov't  
\*Viral Proteins: AN, . . .

L37 ANSWER 77 OF 83 MEDLINE on STN

89269091. PubMed ID: 2543161. **Flaviviruses** can mediate fusion from without in *Aedes albopictus* mosquito cell cultures. Summers P L; Cohen W H; Ruiz M M; Hase T; Eckels K H. (Department of Biologics Research, Walter Reed Army Institute of Research, Washington 20307-5100. ) Virus research, (1989 Apr) Vol. 12, No. 4, pp. 383-92. Journal code: 8410979. ISSN: 0168-1702. Pub. country: Netherlands. Language: English.

AB **Flavivirus**-induced polykaryocytes were detected in monolayers of *Aedes albopictus* (clone C6/36) mosquito cells as early as 20 min after adsorbing virus to these cells. A high multiplicity of infection with **dengue** (DEN)-1, 2, 3, 4, Japanese encephalitis, and **yellow fever viruses** was required to demonstrate fusion from without (FFWO) with these **flaviviruses**. Optimal conditions for FFWO included exposure of adsorbed virus to pH 6.0 and an incubation temperature of 39 degrees C. DEN-2 monoclonal antibodies to the envelope **E** glycoprotein inhibited cell fusion, whereas monoclonal antibodies to the **prM** and NS1 proteins did not inhibit cell fusion. These results indicate that **flaviviruses** cause FFWO soon after adsorption to C6/36 mosquito cells and the process is most likely mediated by the virion envelope **E** glycoprotein.

TI **Flaviviruses** can mediate fusion from without in *Aedes albopictus* mosquito cell cultures.

Journal code: 8410979. ISSN: 0168-1702.

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CT *Aedes*  
Animals  
Antibodies, Monoclonal: IM, immunology  
Antibodies, Viral: IM, immunology  
\*Cell Fusion  
Cells, Cultured  
\***Flavivirus: PH, physiology**  
Hydrogen-Ion Concentration  
Temperature  
Viral Envelope Proteins: PH, physiology

L37 ANSWER 78 OF 83 MEDLINE on STN

88258474. PubMed ID: 3385407. Partial nucleotide sequence and deduced amino acid sequence of the structural proteins of **dengue** virus type 2, New Guinea C and PUO-218 strains. Gruenberg A; Woo W S; Biedrzycka A; Wright P J. (Department of Microbiology, Monash University, Clayton, Victoria, Australia. ) The Journal of general virology, (1988 Jun) Vol. 69 ( Pt 6), pp. 1391-8. Journal code: 0077340. ISSN: 0022-1317. Pub. country: ENGLAND: United Kingdom. Language: English.

AB The nucleotide sequence and the deduced amino acid sequence for the genes encoding the structural proteins of two strains of **dengue** virus type 2 (DEN-2) were determined from cDNA clones. The genes for C, **prM**(M) and **E** proteins were sequenced for the prototype DEN-2 virus, the New Guinea C strain. Also sequenced were the **prM**(M) and **E** genes of PUO-218. This strain of DEN-2 was isolated during 1980 in Bangkok and had received a limited number of laboratory passages. Comparisons of the newly determined sequences with those published for the Jamaica 1409 and Puerto Rico PR-159 (S1 vaccine candidate) strains revealed a close relationship between New Guinea C virus and both the Jamaica and PUO-218 viruses (greater than 96% similarity in nucleotides of the **E** gene), whereas S1 virus was the most divergent.

TI Partial nucleotide sequence and deduced amino acid sequence of the structural proteins of **dengue** virus type 2, New Guinea C and PUO-218 strains.

SO The Journal of general virology, (1988 Jun) Vol. 69 ( Pt 6), pp. 1391-8. Journal code: 0077340. ISSN: 0022-1317.

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CT Amino Acid Sequence  
Animals  
Base Sequence  
Comparative Study  
DNA, Viral: GE, genetics  
**Dengue Virus: CL, classification**  
\***Dengue Virus: GE, genetics**  
Genes, Viral  
Humans  
Molecular Sequence Data  
Research Support, Non-U.S. Gov't  
\*Viral Proteins: GE, genetics  
Viral Structural Proteins

L37 ANSWER 79 OF 83 MEDLINE on STN

88237578. PubMed ID: 2837017. Translation mapping with the **flavivirus** Kunjin: gene order and anomalies in translation of Ns5. Schrader A P; Westaway E G. (Department of Microbiology, Monash University, Clayton, Victoria, Australia. ) Virus research, (1988 Mar) Vol. 9, No. 4, pp. 323-33. Journal code: 8410979. ISSN: 0168-1702. Pub. country: Netherlands. Language: English.

AB Kunjin (KUN) virus-infected cells were synchronized in translation by reversal of hypertonic inhibition; cells were then pulse-labelled with [35S]methionine. Electrophoretic analyses defined the sequence of



previously to be unique and unrelated. GP44 or NS1 was inadequately labelled for analysis and was assumed to be translated sequentially after **E** in the polyprotein sequence. The relationship of the KUN gene order obtained by translation mapping to that proposed by Rice et al. Science (1985) 229, 726-733 based on the nucleotide sequence of **yellow fever virus** is as follows: KUN: 5'-C.GP20.**E**.GP44.P19.P10.P71.(?).P21.P98-3' YF: 5'-C.**prM**.**E**.NS1.ns2a.ns2b.NS3.ns4a.ns4b. NS5-3'. These results eliminate the ambiguities in identities of the previously hypothetical ns2a, ns2b and ns4b. Although ns4a was not positively identified, a labelled protein of Mr 12,000 to 14,000 was observed in one experiment and it mapped in the appropriate position for ns4a. Variation occurred in translation of NS5 when the hypertonic treatment of 40 min at 37 degrees C was reduced in time or in temperature.

TI Translation mapping with the **flavivirus** Kunjin: gene order and anomalies in translation of Ns5.

SO Virus research, (1988 Mar) Vol. 9, No. 4, pp. 323-33.  
Journal code: 8410979. ISSN: 0168-1702.

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CT Animals  
Capsid: BI, biosynthesis  
\*Capsid: GE, genetics  
\***Flavivirus: GE, genetics**  
\*Genes  
\*Genes, Fungal  
Methionine: ME, metabolism  
Molecular Weight  
Peptide Chain Initiation, Translational  
\*Protein Biosynthesis  
Research Support, Non-U.S. Gov't

L37 ANSWER 80 OF 83 MEDLINE on STN

88089524. PubMed ID: 2826659. Nucleotide and complete amino acid sequences of Kunjin virus: definitive gene order and characteristics of the virus-specified proteins. Coia G; Parker M D; Speight G; Byrne M E; Westaway E G. (Department of Microbiology, Monash University, Clayton, Victoria, Australia. ) The Journal of general virology, (1988 Jan) Vol. 69 ( Pt 1), pp. 1-21. Journal code: 0077340. ISSN: 0022-1317. Pub. country: ENGLAND: United Kingdom. Language: English.

AB A Kunjin (KUN) virus cDNA sequence of 10664 nucleotides was obtained and it encoded a single open reading frame for 3433 amino acids. Partial N-terminal amino acid analyses of KUN virus-specified proteins identified the polyprotein cleavage sites and the definitive gene order. The gene order relative to that proposed for yellow fever (YF) virus is as follows: KUN 5'-C.GP20.**E**.GP44.P19.P10.P71.(?).P21.P98-3' YF 5'-C.**prM**.**E**.NS1.ns2a.ns2b.NS3.ns4a.ns4b. NS5-3'. The order of putative signal sequences and stop transfer sequences indicated that KUN NS1, NS2A and NS4B are probably cleaved in the lumen of the endoplasmic reticulum, at a consensus site Val-X-Ala decreases where X is an uncharged residue, and NS2B, NS3 and NS5 are cleaved in the cytosol at the site Lys-Arg decreases Gly. Comparisons with the complete amino acid sequences of YF and West Nile (WN) viruses showed that KUN virus shared 93% homology with WN virus, but only 46% homology with YF virus. Comparisons among individual gene products of six **flaviviruses** showed that **E**, NS1, NS3 and NS5 tended to be the most highly conserved, and C among the least conserved. Homologous cleavage sites were evident, and six domains in NS5, a total of over 170 residues, shared at least 85% homology. Comparisons with the KUN C to NS2B sequence defined a gradient of relationships of all gene products in decreasing order WN greater than Murray Valley greater than Japanese encephalitis greater than St Louis encephalitis viruses within this closely related serological complex. A non-coding 5' sequence (75 nucleotides) of KUN virus shared 95% homology with WN virus and a shorter imperfect match with Murray Valley encephalitis virus (15 of 18 nucleotides). The KUN non-coding 3' sequence of 290 nucleotides contained several short and imperfectly matched sequences, and shared 87% homology over the distal region of 191 nucleotides with the corresponding region of WN virus RNA.

SO The Journal of general virology, (1988 Jan) Vol. 69 ( Pt 1), pp. 1-21.  
Journal code: 0077340. ISSN: 0022-1317.

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46% homology with YF virus. Comparisons among individual gene products of six **flaviviruses** showed that **E**, NS1, NS3 and NS5 tended to be the most highly conserved, and C among the least conserved. Homologous cleavage sites. . .

CT . . . Molecular  
Codon: GE, genetics  
Comparative Study  
DNA Restriction Enzymes  
Encephalitis Virus, Japanese: GE, genetics  
Encephalitis Virus, St. Louis: GE, genetics  
**\*Flavivirus: GE, genetics**  
\*Genes, Viral  
Molecular Sequence Data  
RNA, Viral: GE, genetics  
Research Support, Non-U.S. Gov't  
Sequence Homology, Nucleic Acid  
Vero Cells  
\*Viral Proteins: GE, genetics  
West Nile virus: GE, genetics  
**Yellow fever virus: GE, genetics**

L37 ANSWER 81 OF 83 MEDLINE on STN

87122172. PubMed ID: 3027980. Partial nucleotide sequence of St. Louis encephalitis virus RNA: structural proteins, NS1, ns2a, and ns2b. Trent D W; Kinney R M; Johnson B J; Vorndam A V; Grant J A; Deubel V; Rice C M; Hahn C. Virology, (1987 Feb) Vol. 156, No. 2, pp. 293-304. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB cDNA clones of the St. Louis encephalitis (SLE) virus genome have been obtained and the nucleotide sequence of 4.7 kb corresponding to the 5' terminal half of the genome determined. The genome contains a 5' noncoding region of 98 nucleotides followed by a single continuous open reading frame that encodes three structural proteins in the order capsid (C), membrane precursor (**prM**)-membrane (M), and envelope (**E**). Immediately following the C-terminus of **E** are located nonstructural proteins NS1 through NS3. The SLE amino acid sequence homology with yellow fever (YF), Murray Valley encephalitis (MVE), West Nile (WN), and **dengue-2** (DEN) viruses over the sequenced region is 39, 66, 64, and 43%, respectively. The start of each SLE protein has been assigned on the basis of N-terminal sequence data and potential proteolytic cleavage sites homologous with YF and MVE viruses. **Flaviviruses** have conserved glycosylation sites in **prM** and NS1 proteins, although only one of the two glycosylation sites in the SLE **E** protein is conserved in MVE and DEN viruses. An evolutionary tree showing relationships of SLE, MVE, WN, YF, and DEN-2 **flaviviruses** is proposed on the basis of the amino acid sequences of the C proteins.

SO Virology, (1987 Feb) Vol. 156, No. 2, pp. 293-304.  
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CT Base Sequence  
Capsid: GE, genetics  
Cloning, Molecular  
Comparative Study  
\*Encephalitis Virus, St. Louis: GE, genetics  
Evolution  
**\*Flavivirus: GE, genetics**  
Genes, Viral  
Membrane Proteins: GE, genetics  
Protein Precursors: GE, genetics  
\*RNA, Viral: GE, genetics  
Species Specificity  
Viral Envelope. . .

L37 ANSWER 82 OF 83 MEDLINE on STN

87071658. PubMed ID: 3024394. Nucleotide sequence and deduced amino acid sequence of the structural proteins of **dengue** type 2 virus, Jamaica genotype. Deubel V; Kinney R M; Trent D W. Virology, (1986 Dec) Vol. 155, No. 2, pp. 365-77. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

(Jamaica genotype) virus has been determined and the encoded proteins compared with those of yellow fever and West Nile viruses, which belong to different **flavivirus** serogroups. The cDNA clone which was sequenced contains a 5'-noncoding region of 96 nucleotides followed by a single open reading frame coding for the structural proteins 5'-C-**prM**(M)-**E**-3' and the beginning of the NS1 nonstructural protein. The amino acid sequence homology between the structural polyprotein precursor of **dengue** 2 virus and those of yellow fever and West Nile viruses is 36.5 and 42%, respectively. The **dengue** virus structural proteins are similar in size and composition to those of the other **flaviviruses**. The basic capsid protein and the membrane and envelope proteins have hydrophobic regions at their C termini. The **dengue** 2 capsid C, membrane M, and envelope **E** proteins share 13, 36, and 43% homology, respectively, with the cognate proteins of **yellow fever virus**, and 33, 32, and 47% homology with the cognate proteins of West Nile virus. All 6 cysteine residues in the **dengue** 2 premembrane protein and all 12 cysteine residues in the **dengue** 2 envelope protein are conserved in the cognate proteins of yellow fever and West Nile viruses.

TI Nucleotide sequence and deduced amino acid sequence of the structural proteins of **dengue** type 2 virus, Jamaica genotype.

SO Virology, (1986 Dec) Vol. 155, No. 2, pp. 365-77.

Journal code: 0110674. ISSN: 0042-6822.

AB The nucleotide sequence of the 5'-terminal 2469 bases of **dengue** 2 (Jamaica genotype) virus has been determined and the encoded proteins compared with those of yellow fever and West Nile viruses, which belong to different **flavivirus** serogroups. The cDNA clone which was sequenced contains a 5'-noncoding region of 96 nucleotides followed by a single open reading frame coding for the structural proteins 5'-C-**prM**(M)-**E**-3' and the beginning of the NS1 nonstructural protein. The amino acid sequence homology between the structural polyprotein precursor of **dengue** 2 virus and those of yellow fever and West Nile viruses is 36.5 and 42%, respectively. The **dengue** virus structural proteins are similar in size and composition to those of the other **flaviviruses**. The basic capsid protein and the membrane and envelope proteins have hydrophobic regions at their C termini. The **dengue** 2 capsid C, membrane M, and envelope **E** proteins share 13, 36, and 43% homology, respectively, with the cognate proteins of **yellow fever virus**, and 33, 32, and 47% homology with the cognate proteins of West Nile virus. All 6 cysteine residues in the **dengue** 2 premembrane protein and all 12 cysteine residues in the **dengue** 2 envelope protein are conserved in the cognate proteins of yellow fever and West Nile viruses.

CT Amino Acid Sequence

Base Sequence

Cloning, Molecular

Codon

Comparative Study

DNA: GE, genetics

**Dengue Virus: CL, classification**

**\*Dengue Virus: GE, genetics**

**Flavivirus: GE, genetics**

Protein Conformation

Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, P.H.S.

Solubility

**\*Viral Proteins: GE, genetics**

L37 ANSWER 83 OF 83 MEDLINE on STN

86200215. PubMed ID: 3009829. Partial nucleotide sequence of the Murray Valley encephalitis virus genome. Comparison of the encoded polypeptides with **yellow fever virus** structural and non-structural proteins. Dalgarno L; Trent D W; Strauss J H; Rice C M. Journal of molecular biology, (1986 Feb 5) Vol. 187, No. 3, pp. 309-23. Journal code: 2985088R. ISSN: 0022-2836. Pub. country: ENGLAND: United Kingdom. Language: English.

AB The sequence of 5400 bases corresponding to the 5'-terminal half of the Murray Valley encephalitis virus genome has been determined. The genome contains a 5' non-coding region of about 97 nucleotides, followed by a single continuous open reading frame that encodes the structural proteins followed by the non-structural proteins. Amino acid sequence homology between the Murray Valley encephalitis and yellow fever (Rice et al., 1985) polyproteins is 42% over the region sequenced. The start points of the various Murray Valley encephalitis virus-coded proteins have been assigned on the basis of this homology and a consistent set of potential proteolytic cleavage sites identified, the sequences of which are similar in Murray Valley encephalitis and yellow fever. The deduced Murray Valley encephalitis gene order is 5'-C-**prM**(M)-**E**-NS1-ns2a-ns2b-NS3-3'. The genome organization of Murray Valley encephalitis and yellow fever appears to be identical and the sizes of the predicted virus-coded proteins similar between the two viruses. Both viruses encode a basic capsid protein followed by three glycoproteins; the glycoproteins appear to have the conventional topology of N terminus outside with a C-terminal membrane-spanning domain. There are conserved glycosylation sites in

- non-structural protein of uncertain function. The glycosylation sites in **E**, the major envelope protein of the virion, are not conserved as to position. We predict the existence, in **flavivirus**-infected cells, of two small, hydrophobic peptides, ns2a and ns2b, which show only limited amino acid sequence homology. Finally, about half of the amino acid sequence of NS3 has been obtained; NS3 is a hydrophilic non-structural protein that shows 55% amino acid sequence similarity between Murray Valley encephalitis and yellow fever over the region sequenced and is probably involved in RNA replication.
- TI Partial nucleotide sequence of the Murray Valley encephalitis virus genome. Comparison of the encoded polypeptides with **yellow fever virus** structural and non-structural proteins.
- SO Journal of molecular biology, (1986 Feb 5) Vol. 187, No. 3, pp. 309-23. Journal code: 2985088R. ISSN: 0022-2836.
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- CT Amino Acid Sequence  
Base Sequence  
Capsid  
Codon  
Comparative Study  
**\*Flavivirus: GE, genetics**  
**\*Genes, Viral**  
**\*RNA, Viral**  
Research Support, U.S. Gov't, Non-P.H.S.  
Research Support, U.S. Gov't, P.H.S.  
Terminator Regions (Genetics)  
Viral Proteins: GE, genetics  
Viral Structural Proteins  
**Yellow fever virus: GE, genetics**

=> d 137,cbib,ab,50-70

- L37 ANSWER 50 OF 83 MEDLINE on STN  
94082440. PubMed ID: 8259646. Structural changes and functional control of the tick-borne encephalitis virus glycoprotein **E** by the heterodimeric association with protein **prM**. Heinz F X; Stiasny K; Puschner-Auer G; Holzmann H; Allison S L; Mandl C W; Kunz C. (Institute of Virology, University of Vienna, Austria. ) Virology, (1994 Jan) Vol. 198, No. 1, pp. 109-17. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.
- AB We have used tick-borne encephalitis virus to study the involvement of acidic compartments during the entry and release phases of **flavivirus** infection and to elucidate the role of protein **prM** in immature virions. Elevation of the pH in acidic intracellular compartments by either bafilomycin A1, a specific inhibitor of the vacuolar type H(+)-ATPase or by NH4Cl had a strong inhibitory effect during virus penetration and also prevented the cleavage of **prM** when added in the late phase of the viral life cycle. In the latter case the release of virus particles was not impaired. These immature (**prM**-containing) virions exhibited a 20- to 50-fold lower specific infectivity and HA activity than mature virions and in contrast to these did not undergo low pH-triggered aggregation. The presence of **prM** also affected the binding of monoclonal antibodies to protein **E**, especially at sites which have been shown to undergo acid pH-induced conformational changes in mature virions. Crosslinking, solubilization, and sedimentation analyses revealed the existence of **prM-E** heterooligomeric complexes, suggesting that the function of **prM** is to protect protein **E** from undergoing the irreversible conformational changes in acidic compartments of the secretory pathway that are necessary for triggering fusion activity in the endosome during virus entry.
- L37 ANSWER 51 OF 83 MEDLINE on STN  
94058666. PubMed ID: 8240004. Genetic and biological differentiation of **dengue** 3 isolates obtained from clinical cases in Java, Indonesia, 1976-1978. Lee E; Gubler D J; Weir R C; Dalgarno L. (Division of Biochemistry and Molecular Biology, School of Life Sciences, Australian National University, Canberra. ) Archives of virology, (1993) Vol. 133, No. 1-2, pp. 113-25. Journal code: 7506870. ISSN: 0304-8608. Pub. country: Austria. Language: English.

a series of outbreaks of **dengue** fever and **dengue** haemorrhagic fever/**dengue** shock syndrome which occurred in Java, Indonesia in 1976-1978. In the current study we compare growth characteristics in cell culture, and nucleotide sequence data for the viral **prM** and **E** genes, of five low passage DEN-3 isolates obtained during these epidemics from clinically defined cases. All isolates had the same passage history: human sera were passed twice in mosquitoes and three times in a mosquito cell line (*Aedes albopictus*, C 6/36 cells). Growth differences were observed between individual isolates in Vero cells; growth differences were not observed in C 6/36 cells. Nucleotide sequencing of the **prM** and **E** gene region indicated that no two isolates were identical (sequence divergence ranged from 0.4 to 1.6% in pairwise comparisons) but that they were closely enough related to present a single genetic type. There were one or two differences in deduced amino acid sequence in **E** between isolates. Differences were at residues 65, 187, 298 or 443. One isolate differed from all others at residue 16 in the M protein. No relationship was apparent between the amino acid sequence of M or **E** and the nature of the disease profile, the year of isolation or the geographic region of isolation. The isolates showed 3.5 to 4.4% nucleotide sequence divergence from the highly-adapted H 87 prototype, isolated in the Philippines in 1956. The isolates showed a total of twelve common amino acid differences in **prM** and **E** proteins from H 87. Ten of these twelve residues were at positions which differed between the four **dengue** serotypes. Two differences (at residues 37 in M and 293 in **E**) were at positions which are conserved in sequence between the four **dengue** serotypes. The data are discussed in relation to the **dengue** outbreaks in Java in the period 1976-1978.

L37 ANSWER 52 OF 83 MEDLINE on STN

93314963. PubMed ID: 8325506. Role of protein conformation in the processing of **dengue** virus type 2 nonstructural polyprotein precursor. Zhang L; Padmanabhan R. (Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City 66103. ) Gene, (1993 Jul 30) Vol. 129, No. 2, pp. 197-205. Journal code: 7706761. ISSN: 0378-1119. Pub. country: Netherlands. Language: English.

AB The **dengue** virus type-2 (DEN-2) genome is a positive-strand RNA encoding a single polyprotein precursor, C-**prM**(M)-**E**-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5, consisting of 3391 amino acids (aa). The N-terminal region of the polyprotein precursor, C-**prM**(M)-**E**, encodes the structural proteins and is processed cotranslationally by the host signal peptidase. The nonstructural region NS1-->NS5 is processed by the viral protease(s), as well as by the signal peptidase. A two-component viral protease consisting of NS2B and the serine protease domain of NS3 has been shown to be required for cleavages having the consensus sequence of dibasic aa (K-R, R-R, R-K, or Q-R). In this study, the region encoding all the nonstructural proteins, NS1-->NS5, was expressed using a recombinant vaccinia virus system. Cleavages at the consensus viral protease recognition sites, 2B-3 at the N terminus and 3-4A at the C terminus, are prerequisites to the release of mature NS3 protease. Although the 2B-3 site was cleaved readily in a variety of polyprotein precursors containing the intact NS2B and the NS3 protease domain, the 3-4A site was most efficiently cleaved, similar to that found in DEN-2-infected cells, only in the polyprotein precursor encoding the entire nonstructural region. Removal of NS1 at the N terminus or of NS5 coding sequences at the C terminus affected the cleavage at the 3-4A site to produce the processing intermediate, NS3-NS4A. These results indicate that the conformation of the nonstructural polyprotein precursor, NS1-->NS5, plays a major role in the efficient cleavage at the 3-4A site.

L37 ANSWER 53 OF 83 MEDLINE on STN

93267778. PubMed ID: 8388499. Processing of the envelope glycoproteins of pestiviruses. Rumenapf T; Unger G; Strauss J H; Thiel H J. (Division of Biology, California Institute of Technology, Pasadena 91125. ) Journal of virology, (1993 Jun) Vol. 67, No. 6, pp. 3288-94. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB The genomic RNA of pestiviruses is translated into a large polyprotein that is cleaved into a number of proteins. The structural proteins are N terminal in this polyprotein and include three glycoproteins called E0, E1, and E2 on the basis of the order in which they appear in the polyprotein. Using pulse-chase experiments, we show that a pestiviral glycoprotein precursor, E012, is formed that is processed into E0, E1, and E2 in an ordered fashion. Processing is initiated by a nascent cleavage between the capsid and the translocated E012 followed by cleavage at the C terminus of E2. E012 is then rapidly cleaved to form E01 and E2. After E2 is released from the precursor, E01 is processed into E0 and E1. To identify the sites of cleavage, the N termini of the glycoproteins of the pestivirus classical swine fever virus (formerly termed hog cholera virus) were sequenced after expression in the vaccinia virus system. The N termini are Glu-268 for E0 (gp44/48), Leu-495 for E1 (gp33) and Arg-690 for E2 (gp55). The sequences around the cleavage sites capsid/E0 and E1/E2 conform to the rules known for cellular signal proteases, as does the sequence at the presumed C terminus of E2. The sequence upstream of

processing sites but lacks the typical hydrophobic signal peptide; this cleavage site has characteristics in common with a site in **flaviviruses** that is also cleaved in a delayed fashion. The absence of any membrane-spanning region results in the shedding of E0 by infected cells, and E0 can be detected in the virus-free supernatant. Comparison of the sequences around the cleavage sites of pestiviruses suggests a general processing scheme for the structural glycoproteins. Comparison of the pesti- and **flaviviral** structural glycoproteins suggests analogies between E012 and **prM-E**.

L37 ANSWER 54 OF 83 MEDLINE on STN

93242750. PubMed ID: 8480420. Selection and partial characterization of **dengue 2** virus mutants that induce fusion at elevated pH. Guirakhoo F; Hunt A R; Lewis J G; Roehrig J T. (Division of Vector-Borne Infectious Diseases, Centers for Disease Control, Fort Collins, Colorado 80522. ) *Virology*, (1993 May) Vol. 194, No. 1, pp. 219-23. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB Two types of **dengue** (DEN) 2 virus mutants were selected either by repeated exposure to acidic pH (acid mutant, AM), or by the addition of ammonium chloride to *Aedes albopictus* C6/36 cells prior to and during viral infection (fusion mutant, FM). Both mutants grew more slowly than the parent strain and induced smaller plaques in Vero cells. The 50% fusion from within index for both mutants occurred at least 0.65 pH units higher than with the wild-type DEN virus. A single amino acid substitution (Asn-153 to Asp) was found in the envelope (**E**)-glycoprotein of the AM virus. Three amino acid substitutions were detected on the **E**-glycoprotein of the FM virus: Ile-6 to Met, Asn-134 to Ser, and Asn-153 to Tyr. No mutations were found in the precursor to the membrane protein, **prM**. The DEN virus **E**-glycoprotein has two potential glycosylation sites: Asn-67 and Asn-153. The loss of the potential glycosylation site at Asn-153 or the change in the chemical characteristics resultant from the amino acid substitutions in both mutants implicates these regions of the **E**-glycoprotein in virus-mediated membrane fusion.

L37 ANSWER 55 OF 83 MEDLINE on STN

93172390. PubMed ID: 8437237. Proper maturation of the **Japanese encephalitis virus** envelope glycoprotein requires cosynthesis with the premembrane protein. Konishi E; Mason P W. (Department of Medical Zoology, Kobe University School of Medicine, Japan. ) *Journal of virology*, (1993 Mar) Vol. 67, No. 3, pp. 1672-5. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB The role of the **Japanese encephalitis virus** (JEV) premembrane (**prM**) protein in maturation of the envelope (**E**) glycoprotein was evaluated by using recombinant vaccinia viruses encoding **E** in the presence (vP829) or absence (vP658) of **prM**. Immunofluorescence analyses showed that **E** appeared to be localized in the endoplasmic reticulum of cells infected with JEV, vP829, or vP658. However, reactivity with monoclonal antibodies and behavior in Triton X-114 indicated that **E** produced in the absence of **prM** behaved abnormally. Furthermore, **E** produced in the presence of **prM** by recombinant vaccinia viruses could be incorporated into **flavivirus** pseudotypes, whereas **E** synthesized in the absence of **prM** could not. These results demonstrate that cosynthesis of **prM** is required for proper folding, membrane association, and assembly of the **flavivirus E** protein.

L37 ANSWER 56 OF 83 MEDLINE on STN

93134792. PubMed ID: 8421896. High-level expression of the **Japanese encephalitis virus E** protein by recombinant vaccinia virus and enhancement of its extracellular release by the NS3 gene product. Sato T; Takamura C; Yasuda A; Miyamoto M; Kamogawa K; Yasui K. (Biological Science Laboratory, Nippon Zeon Company, Limited, Kanagawa, Japan. ) *Virology*, (1993 Feb) Vol. 192, No. 2, pp. 483-90. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB Recombinant vaccinia viruses expressing the **prM** and **E** genes of the **Japanese encephalitis virus** (JEV) were constructed by use of synthetic promoters. While the recombinant virus mOJ6-SL, with an optimized vaccinia late-gene promoter, produced a 20-fold elevated level of **E** protein, as well as an 86-kDa precursor protein in infected cells, no significant quantitative difference was detected between the extracellular or cell-surface **E** protein produced by mOJ6-SL and those produced by mOJ6 with the 7.5-kDa promoter. However, when the cells were infected with **Dengue 2** virus before infection with mOJ6-SL, the amount of the extracellular **E** protein increased 16-fold. In addition, enhancement of its extracellular release was observed when cells were co-infected with mOJ6-SL and recombinant vaccinia virus expressing the NS3 gene of JEV.

L37 ANSWER 57 OF 83 MEDLINE on STN

93079894. PubMed ID: 1280384. The Murray Valley encephalitis virus **prM** protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of **E** glycoprotein. Guirakhoo

Centers for Disease Control, Fort Collins, Colorado 80522. ) Virology, (1992 Dec) Vol. 191, No. 2, pp. 921-31. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

- AB To study the role of the precursor to the membrane protein (**prM**) in **flavivirus** maturation, we inhibited the proteolytic processing of the Murray Valley encephalitis (MVE) virus **prM** to membrane protein in infected cells by adding the acidotropic agent ammonium chloride late in the virus replication cycle. Viruses purified from supernatants of ammonium chloride-treated cells contained **prM** protein and were unable to fuse C6/36 mosquito cells from without. When ammonium chloride was removed from the cells, both the processing of **prM** and the fusion activity of the purified viruses were partially restored. By using monoclonal antibodies (MAbs) specific for the envelope (**E**) glycoprotein of MVE virus, we found that at least three epitopes were less accessible to their corresponding antibodies in the **prM**-containing MVE virus particles. Amino-terminal sequencing of proteolytic fragments of the **E** protein which were reactive with sequence-specific peptide antisera or MAb enabled us to estimate the site of the **E** protein interacting with the **prM** to be within amino acids 200 to 327. Since **prM**-containing viruses were up to 400-fold more resistant to a low pH environment, we conclude that the **E-prM** interaction might be necessary to protect the **E** protein from irreversible conformational changes caused by maturation into the acidic vesicles of the exocytic pathway.

L37 ANSWER 58 OF 83 MEDLINE on STN

93055210. PubMed ID: 1331144. Detection of **flavivirus** antigens in purified infected Vero cell plasma membranes. Ng M L; Choo W K; Ho Y L. (Department of Microbiology, National University of Singapore, Kent Ridge. ) Journal of virological methods, (1992 Sep) Vol. 39, No. 1-2, pp. 125-38. Journal code: 8005839. ISSN: 0166-0934. Pub. country: Netherlands. Language: English.

- AB The types of Kunjin virus-specified proteins present in purified Vero cell plasma membrane were studied. Immunofluorescence of unfixed Kunjin virus-infected whole cell monolayers, indicated that two structural proteins (envelope and **prM**) and three non-structural proteins (NS1, 3 and 5) were found at the plasma membrane. There was no obvious progressive accumulation of the observed antigens over the time periods between 8 to 24 h p.i. Thus SDS-PAGE analysis was performed using purified radiolabelled Vero cell plasma membranes. From the protein profiles, all five antigens detected by immunofluorescent staining were also present. In addition, two smaller molecular weight non-structural proteins NS4B and NS2B were also observed. Generally, all the non-structural proteins found in the purified plasma membranes were of the same molecular weights as those found in infected whole cell lysate. Interestingly, both the structural proteins, i.e., envelope (**E**) and **prM** proteins in the plasma membrane sample were of higher molecular weights as compared to the counterparts in the infected whole cell lysate. The envelope protein of purified extracellular Kunjin virus was also lower in molecular weight compared to the same protein in the plasma membrane.

L37 ANSWER 59 OF 83 MEDLINE on STN

92410626. PubMed ID: 1326813. A highly attenuated host range-restricted vaccinia virus strain, NYVAC, encoding the **prM**, **E**, and NS1 genes of **Japanese encephalitis virus** prevents JEV viremia in swine. Konishi E; Pincus S; Paoletti E; Laegreid W W; Shope R E; Mason P W. (Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut 06510. ) Virology, (1992 Sep) Vol. 190, No. 1, pp. 454-8. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

- AB A highly attenuated strain of vaccinia virus (NYVAC) was engineered to express the **Japanese encephalitis virus** (JEV) **prM**, **E**, and NS1 genes or the **prM** and **E** genes. The recombinant viruses were tested as vaccine candidates in pigs, a natural host of JEV. JEV-neutralizing and hemagglutination-inhibiting antibodies appeared in swine sera 7 days after immunization with 10(8) PFU of the recombinant viruses and increased after a second dose at 28 days. The JEV levels detected in the serum after JEV challenge (d56) of the swine with 2 x 10(5) PFU of JEV were significantly reduced in animals inoculated with the recombinant viruses. These results demonstrate the ability of these NYVAC-vectored recombinants to protect pigs from JEV viremia.

L37 ANSWER 60 OF 83 MEDLINE on STN

92260629. PubMed ID: 1349926. Analysis of murine major histocompatibility complex class II-restricted T-cell responses to the **flavivirus** Kunjin by using vaccinia virus expression. Kulkarni A B; Mullbacher A; Parrish C R; Westaway E G; Coia G; Blanden R V. (Division of Cell Biology, John Curtin School of Medical Research, Australian National University, Canberra. ) Journal of virology, (1992 Jun) Vol. 66, No. 6, pp. 3583-92. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

- AB The present paper analyzes the influence of major histocompatibility complex (MHC) class II (I<sub>r</sub>) genes on MHC class II-restricted T-cell

Kunjin virus antigens and identifies the immunodominant Kunjin virus antigens. Generally, mice were primed by intravenous infection with WNV or Kunjin virus, and their CD4+ T cells were stimulated in vitro 14 days later with WNV or Kunjin virus antigens to pulse macrophage or B-cell antigen-presenting cells (APC). WNV-specific in vitro T-cell responses from H-2b mice were higher than those from H-2d, H-2k, and H-2q mice. When recombinant vaccinia virus-derived Kunjin virus antigen preparations were tested in vitro, Kunjin virus-immune T cells of H-2b haplotype responded most strongly to structural (**prM**, **C**, **E**) and membrane-associated nonstructural (NS1) proteins encoded by VKV 1031 and showed weaker responses to cytosolic nonstructural protein NS5 (VKV 1022), whereas the responders of H-2k haplotype responded most strongly to the antigens encoded by VKV 1022 and gave lesser responses to VKV 1031. H-2d T cells gave weaker responses than either H-2b or H-2k cells, with responses to VKV 1031 generally being higher than those to VKV 1022. Responses to VKV 1023 or VKV 1024 encoding all of the NS3 to NS5 gene sequence or to VKV 1023 encoding all of NS3 were weak or absent. Within a given inbred strain, B cells and macrophages differed in their abilities to present recombinant vaccinia virus-derived Kunjin virus antigens, both in terms of magnitude of T-cell responses induced and the particular Kunjin virus protein presented. T cells from different non-MHC genetic backgrounds varied in their requirements of macrophage numbers as APC for maximum reactivity, suggesting that the concentration of class II MHC antigens and other molecules affecting APC-T-cell interaction varied in mice with different genetic backgrounds. Regardless of MHC haplotype, responses to VKV 1024, which encompasses VKV 1023 and VKV 1022, were either absent or lower than those to VKV 1022, possibly reflecting differences in the processing requirements of these two proteins. When mice were primed intravenously with recombinant vaccinia virus and when their CD4+ T cells were stimulated in vitro with native Kunjin virus antigens, VKV 1031 primed more efficiently than Kunjin virus and VKV 1022 primed similarly to Kunjin virus.

L37 ANSWER 61 OF 83 MEDLINE on STN

92188532. PubMed ID: 1312269. Comparison of a **dengue-2** virus and its candidate vaccine derivative: sequence relationships with the **flaviviruses** and other viruses. Blok J; McWilliam S M; Butler H C; Gibbs A J; Weiller G; Herring B L; Hemsley A C; Aaskov J G; Yoksan S; Bhamarapravati N. (Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital, Brisbane, Queensland, Australia. ) *Virology*, (1992 Apr) Vol. 187, No. 2, pp. 573-90. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB A comparison of the sequence of the **dengue-2** 16681 virus with that of the candidate vaccine strain (16681-PDK53) derived from it identified 53 of the 10,723 nucleotides which differed between the strains. Nucleotide changes occurred in genes coding for all virion and nonvirion proteins, and in the 5' and 3' untranslated regions. Twenty-seven of the nucleotide changes resulted in amino acid alterations. The greatest amino acid sequence differences in the virion proteins occurred in **prM** (2.20%; 2/91 amino acids) followed by the M protein (1.33%; 1/75 amino acids), the C protein (0.88%; 1/114 amino acid), and the **E** protein (0.61%; 3/495 amino acids). Differences in the amino acid sequence of nonvirion proteins ranged from 1.51% (6/398 amino acids) in NS4 to 0.33% (3/900 amino acids) in NS5. The encoded protein sequences of 16681-PDK53 were also compared with the published sequences of other **flaviviruses** to obtain a detailed classification of 17 **flaviviruses** using the neighbor-joining tree method. The analyses of the sequence data produced dendrograms which supported the traditional groupings based on serological evidence, and they suggested that the **flaviviruses** have evolved by divergent mutational change and there was no evidence of genetic recombination between members of the group. Comparisons of the sequences of the **flavivirus** polymerase and helicase-like proteins (NS5 and NS3, respectively) with those from other viruses yielded a classification of the **flaviviruses** indicating that the primary division of the **flaviviruses** was between those transmitted by mosquitoes and those transmitted by ticks.

L37 ANSWER 62 OF 83 MEDLINE on STN

92142515. PubMed ID: 1736531. Recombinant vaccinia virus producing the **prM** and **E** proteins of **yellow fever virus** protects mice from lethal yellow fever encephalitis. Pincus S; Mason P W; Konishi E; Fonseca B A; Shope R E; Rice C M; Paoletti E. (Virogenetics Corporation, Troy, New York 12180. ) *Virology*, (1992 Mar) Vol. 187, No. 1, pp. 290-7. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB Four recombinant vaccinia viruses were constructed for expression of different portions of the 17D **yellow fever virus** (YFV-17D) open reading frame. A recombinant, vP869, expressing **prM** and **E** induced high titers of neutralizing and hemagglutination inhibiting antibodies in mice and was protective against intracranial challenge with the French ; neurotropic strain of YFV. Levels of protection were equivalent to those achieved by immunization with the YFV-17D vaccine virus. Recombinant



failed to protect mice against challenge with YFV despite eliciting antibodies to NS1. The vP869-infected HeLa cells produced a particulate extracellular hemagglutinin (HA) similar to that produced by YFV-infected cells, supporting previous studies with **Japanese encephalitis virus** (Mason et al., 1991), suggesting that the ability of recombinant vaccinia virus to produce extracellular HA particles is important for effective **flavivirus** immunity.

L37 ANSWER 63 OF 83 MEDLINE on STN

92109578. PubMed ID: 1729986. Processing of **dengue** virus type 2 structural proteins containing deletions in hydrophobic domains. Gruenberg A; Wright P J. (Department of Microbiology, Monash University, Clayton, Victoria, Australia. ) Archives of virology, (1992) Vol. 122, No. 1-2, pp. 77-94. Journal code: 7506870. ISSN: 0304-8608. Pub. country: Austria. Language: English.

AB The 5' end of the genome of the **dengue** virus type 2 encoding the structural proteins was expressed using recombinant vaccinia virus. Three additional recombinants derived by deletion of selected **dengue** sequences within the parental construct were also expressed. They were designed to assess the role of hydrophobic domains in the processing of the viral polyprotein in intact cells. The first construct contained a deletion of nucleotides encoding most of the C protein; nucleotides encoding the hydrophobic domain at the carboxy terminus were retained. The second and third constructs contained smaller deletions of 72 bp and 129 bp encoding hydrophobic domains at the carboxy termini of C and **prM** respectively. Indirect immunofluorescence and radioimmunoprecipitation were used to detect **prM** and **E** in cells infected with recombinant viruses. The results showed that deletion of 90% of C had no apparent effect on the processing of **prM** and **E**, and that the signal sequence for **E** at the carboxy terminus of **prM** was active in the absence of the upstream signal sequence for **prM** at the carboxy terminus of C. Deletion of the hydrophobic sequences preceding the amino terminus of **E** prevented cleavage at the **prM-E** junction. These results obtained using infected cells were consistent with the published findings for the translation of **flavivirus** RNA in vitro, and indicated the importance of membrane association in the cleavage of structural proteins from the **flavivirus** polyprotein. In addition, cells infected with the recombinant virus containing the large deletion in the C coding region released the **E** glycoprotein into the culture medium.

L37 ANSWER 64 OF 83 MEDLINE on STN

92024099. PubMed ID: 1833876. Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize **E** or NS1 of **Japanese encephalitis virus**. Konishi E; Pincus S; Fonseca B A; Shope R E; Paoletti E; Mason P W. (Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut 06510. ) Virology, (1991 Nov) Vol. 185, No. 1, pp. 401-10. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB Immunization with recombinant vaccinia viruses that specified the synthesis of **Japanese encephalitis virus** (JEV) glycoproteins protected mice from a lethal intraperitoneal challenge with JEV. Recombinants which coexpressed the genes for the structural glycoproteins, **prM** and **E**, elicited high levels of neutralizing (NEUT) and hemagglutination inhibiting (HAI) antibodies in mice and protected mice from a lethal challenge by JEV. Recombinants expressing only the gene for the nonstructural glycoprotein, NS1, induced antibodies to NS1 but provided low levels of protection from a similar challenge dose of JEV. Antibodies to the NS3 protein in postchallenge sera, representing the degree of infection with challenge virus, were inversely correlated to NEUT and HAI titers and levels of protection. These results indicate that although vaccinia recombinants expressing NS1 can provide some protection from lethal JEV infection, recombinants expressing **prM** and **E** elicited higher levels of protective immunity.

L37 ANSWER 65 OF 83 MEDLINE on STN

91280412. PubMed ID: 1965931. Overview of **flavivirus** molecular biology and future vaccine development via recombinant DNA. Rice C M. (Department of Molecular Microbiology, Washington University School of Medicine, St Louis, Missouri 63110-1093. ) The Southeast Asian journal of tropical medicine and public health, (1990 Dec) Vol. 21, No. 4, pp. 670-7. Ref: 54. Journal code: 0266303. ISSN: 0125-1562. Pub. country: Thailand. Language: English.

AB Studies in many laboratories over the last several years have elucidated the structures of several different **flavivirus** genomes. Conserved features include the production of at least 10 different virus encoded proteins from a single long open reading frame by a combination of host and virus-encoded proteases. The established gene order is 5'-C-**prM**(M)-**E**-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS 5-3' and these proteins exhibit varying degrees of homology in comparisons among **flaviviruses**. Conserved RNA sequences and structures have also been identified for the mosquito-borne **flaviviruses** but are absent in sequenced tick-borne viruses. Relevant to the development of efficacious **flavivirus**

for eliciting protective immunity have focused primarily on the structural proteins, in particular the **E** protein, as well as the nonstructural secreted glycoprotein, NS1. Other work, which has led to the derivation of live-attenuated **flavivirus** strains, should eventually allow the genetic determinants of **flavivirus** attenuation and pathogenesis to be understood at the molecular level. The successful recovery infectious **flaviviruses** from cloned cDNA raises the possibility of manipulating these viral genomes as cDNA to construct or propagate candidate live-attenuated vaccine strains. Several applications of this technology are discussed.

L37 ANSWER 66 OF 83 MEDLINE on STN

91280411. PubMed ID: 1711716. Analysis of Japanese encephalitis (JE) virus genome and implications for recombinant JE vaccine. Yasui K; Miyamoto M; Kimura-Kuroda J; Yasuda A; Matsuura Y; Sato T; Kojima A; Kubonoya H. (Department of Microbiology, Tokyo Metropolitan Institute for Neurosciences, Japan. ) The Southeast Asian journal of tropical medicine and public health, (1990 Dec) Vol. 21, No. 4, pp. 663-9. Journal code: 0266303. ISSN: 0125-1562. Pub. country: Thailand. Language: English.

AB From the information of nucleotide sequences and deduced amino acid sequences of **flaviviruses** including JEV, we can postulate processing mechanisms of a polyprotein translated from single long open reading frame of the genome and mechanisms of construction of antigenic structures of structural proteins with biologically active forms after these proteins are translated. The results of comparative analysis of amino acid sequences among **flaviviruses** and epitope analysis on the **E** proteins which are the most important antigens for protective immunity suggest that the **E** protein of **flaviviruses** may have a similar structure closely related to each other. **PrM** and **E** proteins which had predictable signal sequences upstream on the N terminals were expressed with antigenically active form and molecular size the same as the authentic ones by the recombinant viruses. However, the recombinant viruses which had no such signal sequence expressed unprocessed proteins with antigenically denatured forms. These results suggest that normal proteolytic processing is needed to construct biologically active structures of JEV structural proteins. The **E** proteins which were expressed by the recombinant viruses as antigenically active form could elicit neutralizing and HI antibodies in animals and protective immunity in mice. The recombinant vaccinia viruses which express the **E** protein could induce strong immunologic memory against the **E** protein in mice. These results indicate that the development of a new type of vaccine against JEV will become possible in future.

L37 ANSWER 67 OF 83 MEDLINE on STN

91280408. PubMed ID: 2098930. Antigenic analysis of **dengue** virus using monoclonal antibodies. Young P R. (Sir Albert Sakazewski Virus Research Laboratory, Royal Children's Hospital, Brisbane, Australia. ) The Southeast Asian journal of tropical medicine and public health, (1990 Dec) Vol. 21, No. 4, pp. 646-51. Journal code: 0266303. ISSN: 0125-1562. Pub. country: Thailand. Language: English.

AB **Dengue** viruses are classified as a separate antigenic group within the **Flaviviridae** on the basis of cross-reactivity in neutralization assays employing polyclonal sera. Additional serological relationships defining group, complex and type specificity between members of the various antigenic groups have also been identified with polyclonal sera in analyses using hemagglutination inhibition (HI) and complement fixation (CF) tests. With the advent of monoclonal antibodies, however, this picture has become far more complex. While the basic framework of serological relationships has been confirmed, a large number of additional cross-reactivities have been identified that suggest a much greater degree of antigenic diversity and/or relatedness than previously imagined. Monoclonal antibodies have not only been used to dissect the antigenic relatedness between **flaviviruses** but also in studies aimed at defining epitopes on viral proteins involved in a range of biological activities from protection to antibody-dependent enhancement (ADE) of infection. Of the ten proteins encoded by the **dengue** virus genome, monoclonal antibodies have been raised to six, including each of the structural proteins (C, **prM**, **E**) and three of the non-structural proteins (NS1, NS3, NS5). These antibodies have been applied to the construction of functional maps and in particular to the definition of antigenic determinants involved in protection.

L37 ANSWER 68 OF 83 MEDLINE on STN

91259060. PubMed ID: 1710648. Fusion activity of **flaviviruses**: comparison of mature and immature (**prM**-containing) tick-borne encephalitis virions. Guirakhoo F; Heinz F X; Mandl C W; Holzmann H; Kunz C. (Institute of Virology, University of Vienna, Austria. ) The Journal of general virology, (1991 Jun) Vol. 72 ( Pt 6), pp. 1323-9. Journal code: 0077340. ISSN: 0022-1317. Pub. country: ENGLAND: United Kingdom. Language: English.

AB The fusion activity of **flaviviruses** [tick-borne encephalitis (TBE) virus and **Japanese encephalitis virus**] was assessed by inducing fusion

Membrane fusion and polykaryocyte formation was observed only after incubating the viruses at acidic pH. Two groups of monoclonal antibodies reacting with distinct non-overlapping antigenic domains on the TBE virus protein **E** inhibited fusion from without. One of these domains contains the most highly conserved and putative fusion-active sequence of the **flavivirus** protein **E**. Of five TBE virus monoclonal antibody escape mutants, each defined by a single amino acid substitution in the envelope protein **E**, one revealed a reduced fusion activity and another one a lower pH threshold. TBE virus grown in the presence of ammonium chloride as well as Langat virus purified from the supernatant of infected chick embryo cells contained the precursor of protein M (**prM**) rather than M itself. These 'immature' virions did not cause fusion from without, suggesting that the proteolytic processing of **prM** may be necessary for the generation of fusion-competent virions.

L37 ANSWER 69 OF 83 MEDLINE on STN

91111984. PubMed ID: 1824904. The sensitivity of cell-associated **dengue** virus proteins to trypsin and the detection of trypsin-resistant fragments of the nonstructural glycoprotein NS1. Cauchi M R; Henschel E A; Wright P J. (Department of Microbiology, Monash University, Clayton, Victoria, Australia. ) *Virology*, (1991 Feb) Vol. 180, No. 2, pp. 659-67. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB Extracts of Vero cells infected with **dengue** virus type 2 were digested by trypsin in the presence and absence of detergents. The experiments were designed to test the models proposed for **flavivirus** translation in which the glycoproteins **prM**, **E**, and NS1 are inserted into the endoplasmic reticulum of the cell, whereas certain other nonstructural proteins are not. Viral polypeptides were detected by the use of radiolabel, by immunoprecipitation, or by immunoblotting. The results obtained for NS3 and NS5 were as predicted by the models, with membranes providing no protection against digestion by trypsin. Similarly, the results obtained for **prM** and **E** were consistent with the models, with membranes protecting against proteolysis. Some molecules of NS1 were protected, while others were sensitive to proteolysis; novel trypsin-resistant fragments of 69,000, 60,000, and 50,000 Mr (all heat-labile), and of 37,000 and 24,000 Mr were detected following treatment of cell extracts with various combinations of trypsin, detergent, and reducing agent. Preliminary experiments suggested that these tryptic fragments are potentially useful in mapping the antigenic epitopes of NS1.

L37 ANSWER 70 OF 83 MEDLINE on STN

91083519. PubMed ID: 2260923. Expression of the structural proteins of **dengue** 2 virus and **yellow fever virus** by recombinant vaccinia viruses. Hahn Y S; Lenches E M; Galler R; Rice C M; Dalrymple J; Strauss J H. (Division of Biology, California Institute of Technology, Pasadena. ) *Archives of virology*, (1990) Vol. 115, No. 3-4, pp. 251-65. Journal code: 7506870. ISSN: 0304-8608. Pub. country: Austria. Language: English.

AB Vaccinia virus recombinants were constructed which contained cDNA sequences encoding the structural region of **dengue** 2 virus (PR159/S1 strain) or **yellow fever virus** (17D strain). The **flavivirus** cDNA sequences were expressed under the control of the vaccinia 7.5k early/late promoter. Cultured cells infected with these recombinants expressed immunologically reactive **flavivirus** structural proteins, precursor **prM** and **E**. These proteins appeared to be cleaved and glycosylated properly since they comigrated with the authentic proteins from **dengue** 2 virus- and **yellow fever virus**-infected cells. Mice immunized with the **dengue**/vaccinia recombinant showed a **dengue**-specific immune response that included low levels of neutralizing antibodies. Immunization of mice with the **yellow fever**/vaccinia recombinant was less effective at inducing an immune response to **yellow fever virus** and in only some of the mice were low titers of neutralizing antibodies produced.

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L37 ANSWER 26 OF 83 MEDLINE on STN

97288308. PubMed ID: 9143286. Construction of infectious cDNA clones for **dengue** 2 virus: strain 16681 and its attenuated vaccine derivative, strain PDK-53. Kinney R M; Butrapet S; Chang G J; Tsuchiya K R; Roehrig J T; Bhamarapravati N; Gubler D J. (Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado, USA.. rmkl@cdc.gov) . *Virology*, (1997 Apr 14) Vol. 230, No. 2, pp. 300-8. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB We identified nine nucleotide differences between the genomes of **dengue**-2 (DEN-2) 16681 virus and its vaccine derivative, strain PDK-53. These included a C-to-T (16681-to-PDK-53) mutation at nucleotide position 57 of the 5'-untranslated region, three silent mutations, and substitutions **prM**-29 Asp to Val, NS1-53 Gly to Asp, NS2A-181 Leu to Phe, NS3-250 Glu to Val, and NS4A-75 Gly to Ala. Unpassaged PDK-53 vaccine

We constructed infectious cDNA clones for 16681 virus and each of the two PDK-53 variants. DEN-2 16681 clone-derived viruses were identical to the 16681 virus in plaque size and replication in LLC-MK2 cells, replication in C6/36 cells, **E** and **prM** epitopes, and neurovirulence for suckling mice. PDK-53 virus and both clone-derived PDK-53 variants were attenuated in mice. However, the variant containing NS3-250-Glu was less temperature sensitive and replicated better in C6/36 cells than did PDK-53 virus. The variant containing NS3-250-Val had smaller, more diffuse plaques, decreased replication, and increased temperature sensitivity in LLC-MK2 cells relative to PDK-53 virus. Both PDK-53 virus and the NS3-250-Val variant replicated poorly in C6/36 cells relative to 16681 virus. Unpassaged PDK-53 vaccine virus and the virus passaged once in LLC-MK2 cells had genomes of identical sequence, including the mixed NS3-250-Glu/Val locus. Although the NS3-250-Val mutation clearly affected virus replication in vitro, it was not a major determinant of attenuation for PDK-53 virus in suckling mice.

L37 ANSWER 27 OF 83 MEDLINE on STN

97170855. PubMed ID: 9018134. Poxvirus-based Japanese encephalitis vaccine candidates induce JE virus-specific CD8+ cytotoxic T lymphocytes in mice. Konishi E; Kurane I; Mason P W; Shope R E; Ennis F A. (Department of Medical Zoology, Kobe University School of Medicine, Chuo-ku, Japan.. ekon@icluna.kobe-u.ac.jp) . Virology, (1997 Jan 20) Vol. 227, No. 2, pp. 353-60. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB Recombinant Japanese encephalitis (JE) vaccine candidates based on a highly attenuated vaccinia virus (NYVAC-JEV) and a canarypox virus (ALVAC-JEV) were evaluated for their ability to induce specific antibodies and cytotoxic T lymphocytes (CTLs) in mice. Six- to eight-week-old male Balb/c mice that received one or two intraperitoneal inoculations with these JE vaccine candidates at a dose of  $1 \times 10^7$  PFU per mouse produced neutralizing antibody and antibodies to the envelope (**E**) and nonstructural 1 (NS1) proteins as determined by radioimmunoprecipitation. Immunization with either of these vaccine candidates also induced JE virus-specific T lymphocytes that proliferated in response to stimulation with infectious virus and/or noninfectious viral antigens. Mice maintained detectable levels of neutralizing antibody and JE virus-specific memory T cells for at least 6 months after immunization with NYVAC-JEV and for 4 months after immunization with ALVAC-JEV. Cells induced to proliferate after stimulation with live virus contained specific CD8+ CTLs that lysed primary Balb/c mouse kidney cells infected with JE virus and P815 mastocytoma cells infected with a recombinant vaccinia virus expressing the premembrane (**prM**), **E**, and NS1 proteins. These CTLs also lysed P815 cells infected with vaccinia recombinants expressing **prM** and **E**, and those expressing **E** and NS1, but did not lyse P815 cells infected with a recombinant virus expressing only NS1, indicating that the CTLs mainly recognized **E**, but did not recognize NS1. These results demonstrate that both recombinant JE vaccines, NYVAC-JEV and ALVAC-JEV, induce JE virus-specific antibody and CTLs in mice.

L37 ANSWER 28 OF 83 MEDLINE on STN

97155358. PubMed ID: 9002072. Evaluation of monoclonal antibodies for generic detection of **flaviviruses** by ELISA. Brown J M; Coates D M; Phillpotts R J. (Chemical and Biological Defence Establishment, Salisbury, Wiltshire, UK.. 100432.3200@compuserve.com) . Journal of virological methods, (1996 Dec) Vol. 62, No. 2, pp. 143-51. Journal code: 8005839. ISSN: 0166-0934. Pub. country: Netherlands. Language: English.

AB Three monoclonal antibodies (Mabs) specific for the envelope (**E**) protein of **flaviviruses** were evaluated for use in an antigen capture ELISA. Three combinations of Mabs and a combination of polyclonal antibodies (Pabs) were evaluated in antigen capture ELISAs for their ability to detect 18 **flaviviruses**. The Mab ELISAs detected 50% of **flavivirus** antigens with a sensitivity between 1 and  $9 \times 10^4$ /ng viral protein/ml, however, none of the ELISAs evaluated proved to be useful for generic detection of **flaviviruses**, being unable to detect tick-borne **flaviviruses** and some mosquito-borne **flaviviruses**. The inability of the ELISAs to detect tick-borne **flaviviruses** is thought to be due to the conformation of surface epitopes, which the Mabs were unable to recognise. This was again observed using recombinant TBE virus **prM/E** protein as antigen in direct and antigen capture ELISAs. The Mabs reacted with the **prM/E** protein when it was denatured by binding directly onto the solid phase, but the antibodies were unable to detect the native protein in antigen capture ELISAs. The antigen capture ELISAs evaluated in this study were considered to be unsuitable for the generic detection of **flaviviruses**, but may provide a sensitive diagnostic assay for specific **flavivirus** infection.

L37 ANSWER 29 OF 83 MEDLINE on STN

97151139. PubMed ID: 8995675. Subgenomic replicons of the **flavivirus** Kunjin: construction and applications. Khromykh A A; Westaway E G. (Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital, Brisbane, Queensland, Australia.. A.Khromykh@mailbox.uq.oz.au) . Journal

0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.  
AB Several Kunjin virus (KUN) subgenomic replicons containing large deletions in the structural region (C-**prM-E**) and in the 3' untranslated region (3'UTR) of the genome have been constructed. Replicon RNA deltaME with 1,987 nucleotides deleted (from nucleotide 417 [in codon 108] in the C gene to nucleotide 2403 near the carboxy terminus of the **E** gene, inclusive) and replicon RNA C20rep with 2,247 nucleotides deleted (from nucleotide 157 [in codon 20] in C to nucleotide 2403) replicated efficiently in electroporated BHK21 cells. A further deletion from C20rep of 53 nucleotides, reducing the coding sequence in core protein to two codons (C2rep RNA), resulted in abolishment of RNA replication. Replicon deltaME/76 with a deletion of 76 nucleotides in the 3'UTR of deltaME RNA (nucleotides 10423 to 10498) replicated efficiently, whereas replicon deltaME/352 with a larger deletion of 352 nucleotides (nucleotides 10423 to 10774), including two conserved sequences RCS3 and CS3, was significantly inhibited in RNA replication. To explore the possibility of using a reporter gene assay to monitor synthesis of the positive strand and the negative strand of KUN RNA, we inserted a chloramphenicol acetyltransferase (CAT) gene into the 3'UTR of deltaME/76 RNA under control of the internal ribosomal entry site (IRES) of encephalomyelocarditis virus RNA in both plus (deltaME/76CAT[+])- and minus (deltaME/76CAT[-])-sense orientations. Although insertion of the IRES-CAT cassette in the plus-sense orientation resulted in a significant (10- to 20-fold) reduction of RNA replication compared to that of the parental deltaME/76 RNA, CAT expression was readily detected in electroporated BHK cells. No CAT expression was detected after electroporation of RNA containing the IRES-CAT cassette inserted in the minus-sense orientation despite its apparently more efficient replication (similar to that of deltaME/76 RNA); this result indicated that KUN negative-strand RNA was probably not released from its template after synthesis. Replacement of the CAT gene in the deltaME/76CAT(+) RNA with the neomycin gene (Neo) enabled selection and recovery of a BHK cell culture in which the majority of cells were continuously expressing the replicon RNA for 41 days (nine passages) without apparent cytopathic effect. The constructed KUN replicons should provide valuable tools to study **flavivirus** RNA replication as well as providing possible vectors for a long-lasting and noncytopathic RNA virus expression system.

L37 ANSWER 30 OF 83 MEDLINE on STN  
97095957. PubMed ID: 8940981. Immunogenic and protective response in mice immunized with a purified, inactivated, **Dengue-2** virus vaccine prototype made in fetal rhesus lung cells. Putnak R; Cassidy K; Conforti N; Lee R; Sollazzo D; Truong T; Ing E; Dubois D; Sparkuhl J; Gastle W; Hoke C. (Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, District of Columbia, USA. ) The American journal of tropical medicine and hygiene, (1996 Nov) Vol. 55, No. 5, pp. 504-10. Journal code: 0370507. ISSN: 0002-9637. Pub. country: United States. Language: English.

AB The feasibility of a purified, inactivated vaccine (PIV) against **dengue** type 2 (DEN-2) virus was explored. **Dengue-2** virus strain 16681 was used for producing a monotypic PIV. Virus adapted to fetal rhesus lung (FRhL-2) cells was harvested from roller bottle culture supernatant fluids, concentrated, and purified on sucrose gradients. Analysis of purified virus preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting showed primarily envelope (**E**) and premembrane (**prM**) antigens. These preparations had a purity, estimated from silver-stained gels, of approximately 70%, and a yield, based on recovery of virus and viral antigen, of 10-20%. The purified virus was inactivated with 0.05% formalin at 22 degrees C, or alternatively, with 7 mRads from a 60Co source. Vaccinated mice developed high titers of anti-DEN-2 virus neutralizing antibody and were partially protected from virus challenge. These results warrant further testing and development of PIVs for the other DEN virus serotypes.

L37 ANSWER 31 OF 83 MEDLINE on STN  
97037287. PubMed ID: 8882934. Identification of peptides mimicking the antigenicity and immunogenicity of conformational epitopes on **Japanese encephalitis virus** protein using synthetic peptide libraries. Hirabayashi Y; Fukuda H; Kimura J; Miyamoto M; Yasui K. (Department of Microbiology and Immunology, Tokyo Metropolitan Institute for Neuroscience, Japan.. hirabaya@tmin.ac.jp) . Journal of virological methods, (1996 Sep) Vol. 61, No. 1-2, pp. 23-36. Journal code: 8005839. ISSN: 0166-0934. Pub. country: Netherlands. Language: English.

AB Monoclonal antibodies (mAbs) N.03 and N.08 that recognize conformational epitopes on the **prM** protein of **Japanese encephalitis virus** (JEV) were analyzed to identify their peptide ligands by using a novel approach that combined two different synthetic peptide libraries. Immunoscreening of a library containing 20(5) sequences of pentapeptides revealed that the ligands for N.03 and N.08 had motif sequences, (Y/W/F)GG(I/L/M) and (N/Q)WY(D/E), respectively. To select higher-affinity ligands, we synthesized and screened another type of library with 20 peptide mixtures that were based on the identified motif, where only one amino acid

undefined positions. Consequently, the peptides YGGIYMNG and QWYDDR were identified as peptide ligands of N.03 and N.08, respectively. These peptides bound specifically to the antigen-combining sites of the mAbs as confirmed by competitive binding assays. Mouse antisera directed against the peptide YGGIYMNG specifically recognized JEV, while those against QWYDDR did not. These data demonstrated that peptide ligands which reproduce or mimic the immunogenicity as well as the antigenicity of conformational epitopes can be at least partly identified using this approach. This approach may be useful for analyzing conformational epitopes, which are generally difficult to characterize, and might provide a step toward vaccine development when applied to protective mAbs.

L37 ANSWER 32 OF 83 MEDLINE on STN

97017585. PubMed ID: 8864202. Characterization of Langat virus antigenic determinants defined by monoclonal antibodies to E, NS1 and preM and identification of a protective, non-neutralizing preM-specific monoclonal antibody. Iacono-Connors L C; Smith J F; Ksiazek T G; Kelley C L; Schmaljohn C S. (Virology Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21702-5011, USA. ) Virus research, (1996 Aug) Vol. 43, No. 2, pp. 125-36. Journal code: 8410979. ISSN: 0168-1702. Pub. country: Netherlands. Language: English.

AB Hybridomas secreting monoclonal antibodies (MAb) to the tick-borne encephalitis (TBE) group virus, Langat virus (LGTV), were prepared. Of more than 200 MAb screened, 19 antibodies, which cross-reacted with the etiologic agent of Central European encephalitis, were selected for further characterization. Of these MAb, 15 were specific for LGTV E glycoprotein, two for the NS1 protein, and three for preM protein. The two NS1-specific MAb and two of the E-specific MAb reacted with all six of the other TBE group viruses tested while the remainder of the E-specific MAb failed to recognize at least one of the viruses. None of the MAb neutralized LGTV in cell culture assays, but one of the preM-specific MAb protected weanling mice against a virulent LGTV challenge. Although protective antibodies to E and NS1 proteins of TBE viruses were reported, our data provided the first evidence for protection by a non-neutralizing antibody to the preM or M protein of any of the tick-borne **flaviviruses**.

L37 ANSWER 33 OF 83 MEDLINE on STN

96430837. PubMed ID: 8833919. Dominant recognition by human CD8+ cytotoxic T lymphocytes of **dengue** virus nonstructural proteins NS3 and NS1.2a. Mathew A; Kurane I; Rothman A L; Zeng L L; Brinton M A; Ennis F A. (Division of Infectious Diseases and Immunology, Department of Medicine, University of Massachusetts Medical Center, Worcester 01655, USA. ) The Journal of clinical investigation, (1996 Oct 1) Vol. 98, No. 7, pp. 1684-91. Journal code: 7802877. ISSN: 0021-9738. Pub. country: United States. Language: English.

AB A severe complication of **dengue** virus infection, **dengue** hemorrhagic fever (DHF), is hypothesized to be immunologically mediated and virus-specific cytotoxic T lymphocytes (CTLs) may trigger DHF. It is also likely that **dengue** virus-specific CTLs are important for recovery from **dengue** virus infections. There is little available information on the human CD8+ T cell responses to **dengue** viruses. Memory CD8+CTL responses were analyzed to determine the diversity of the T cell response to **dengue** virus and to identify immunodominant proteins using PBMC from eight healthy adult volunteers who had received monovalent, live-attenuated candidate vaccines of the four **dengue** serotypes. All the donors had specific T cell proliferation to **dengue** and to other **flaviviruses** that we tested. CTLs were generated from the stimulated PBMC of all donors, and in the seven donors tested, **dengue** virus-specific CD8+CTL activity was demonstrated. The nonstructural (NS3 and NS1.2a) and envelope (E) proteins were recognized by CD8+CTLs from six, five, and three donors, respectively. All donors recognized either NS3 or NS1.2a. In one donor who received a **dengue** 4 vaccine, CTL killing was seen in bulk culture against the premembrane protein (prM). This is the first demonstration of a CTL response against the **prM** protein. The CTL responses using the PBMC of two donors were serotype specific, whereas all other donors had serotype-cross-reactive responses. For one donor, CTLs specific for E, NS1.2a, and NS3 proteins were all HLA-B44 restricted. For three other donors tested, the potential restricting alleles for recognition of NS3 were B38, A24, and/or B62 and B35. These results indicate that the CD8+CTL responses of humans after immunization with one serotype of **dengue** virus are diverse and directed against a variety of proteins. The NS3 and NS1.2a proteins should be considered when designing subunit vaccines for **dengue**.

L37 ANSWER 34 OF 83 MEDLINE on STN

96400166. PubMed ID: 8806542. Generation and characterization of organ-tropism mutants of **Japanese encephalitis virus** in vivo and in vitro. Chen L K; Lin Y L; Liao C L; Lin C G; Huang Y L; Yeh C T; Lai S C; Jan J T; Chin C. (Institute of Preventive Medicine, National Defense Medical Center, Taipei, Taiwan, Republic of China.. lkchen@ndmcl.ndmctsgh.edu.tw) . Virology, (1996 Sep 1) Vol. 223, No. 1,

States. Language: English.

- AB Using gamma-ray irradiation, a pair of virulent (RP-9) and attenuated (RP-2ms) variants of **Japanese encephalitis virus** (JEV) were generated from a Taiwanese isolate, NT109. The two variants differed in plaque morphology, virus adsorption, and growth properties in BHK-21 cells: (i) RP-2ms produced smaller plaques than RP-9; (ii) RP-2ms adsorbed less efficiently to host cells but yielded a higher virus titer (burst size); and (iii) RP-2ms virions were mostly accumulated intracellularly, whereas RP-9 was released extracellularly. In addition, in an in vitro binding assay, the envelope (**E**) protein of RP-9, but not that of RP-2ms, bound specifically to a cellular protein of 57-kDa derived from BHK-21 cells. When injected into mice intracerebrally, RP-2ms was much less virulent than RP-9, with 50% lethal doses of  $> 10(7)$  and 0.4 plaque forming units, respectively. Moreover, when inoculated intraperitoneally, their organ tropism differed in that the main target organ for RP-2ms was liver, whereas that for RP-9 was brain. These results suggest that RP-2ms was less neurovirulent and less neuroinvasive from peripheral routes. Molecular analysis of the virus structural proteins detected only two differences between RP-9 and RP-2ms: one in **E** protein, Glu-138 in RP-9 and Lys-138 in RP-2ms, and the other in **prM**, Tyr-43 in RP-9 and His-43 in RP-2ms. Since the N-terminal 92 amino acids of **prM** are cleaved and not present in mature JEV virions, the single-amino-acid change of the **E** protein at position 138 may account for the difference between the mutants in the in vitro binding assay. Such mutation in **E** protein, or perhaps in conjunction with the **prM** mutation, may be responsible, in part, for the phenotypic differences observed in vitro and in vivo between the two mutants.

L37 ANSWER 35 OF 83 MEDLINE on STN

96399746. PubMed ID: 8806178. Phylogenetic analysis of the envelope gene of **Japanese encephalitis virus**. Paranjpe S; Banerjee K. (National Institute of Virology, Pune, India. ) Virus research, (1996 Jun) Vol. 42, No. 1-2, pp. 107-17. Journal code: 8410979. ISSN: 0168-1702. Pub. country: Netherlands. Language: English.

- AB Variation among **Japanese encephalitis virus** (JEV) strains has been documented in a number of studies by employing a variety of techniques like HI, NT, CF, RNA fingerprinting and sequencing of **prM** region. We report the complete envelope (**E**) gene sequence and the deduced amino acid sequence of four strains of JEV from the Indian subcontinent. These sequences were compared with published **E** gene sequences of 16 strains of JEV. Pairwise comparisons of the **E** gene nucleotide and deduced amino acid sequences of these strains indicated an overall sequence conservation. A majority of the differences in the four strains were located in domain A and domain C (Mandl et al., 1989). Phylogenetic analysis of the **E** gene sequences by a variety of tree building methods identified four clusters. Viral groupings did not correspond to geographic origin, isolation host or virulence. Evidence for positive selection operating on some strains belonging to different clusters was obtained.

L37 ANSWER 36 OF 83 MEDLINE on STN

96398262. PubMed ID: 8805101. Stability of hemagglutinating activity of extracellular and intracellular forms of **Japanese encephalitis virus** exposed to acidic pH. Nawa M. (Department of Microbiology, Saitama Medical School, Japan. ) Microbiology and immunology, (1996) Vol. 40, No. 5, pp. 365-71. Journal code: 7703966. ISSN: 0385-5600. Pub. country: Japan. Language: English.

- AB The hemagglutinating (HA) activity of extracellular and intracellular forms of Japanese encephalitis (JE) virus was comparatively titrated by exposure to acidic pH below 7.0. A pH-dependent irreversible loss in titer was observed with the virus grown in both C6/36 and BHK 21 (BHK) cells maintained in the pH range of 5.8 to 7.0 for 10 min at 37 C. The HA activity of intracellular virus was relatively more stable than that of extracellular virus in the pH range of 5.8 to 6.4. Virion structural components, envelope glycoprotein (**E**), capsid (C), and membrane (M) proteins in extracellular virus and **E**, C, and the precursor form of M (**prM**) proteins in intracellular virus were detected by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. A panel of monoclonal antibody (mAb) directed for nine antigenic epitopes on the JE virus **E** protein molecule was used for the analysis of antigenic reactivity of **E** protein after treatment at pH 6.0. The reaction between the extracellular virus and three HA-inhibiting (HI) mAbs was significantly reduced after acid treatment; however, the antigenic reactivity of intracellular virus was much more stable with a 100- to 1,000-fold difference. Infectivity titers of extracellular and intracellular viruses in Vero cells were reduced by 1/24,100 and 1/21,666 after acidic treatment at pH 6.0. In contrast, the infectivity of intracellular viruses was more stable, with residual infectivity of 1/182 and 1/340 for BHK and C6/36 cell-grown virus, respectively. Acidic treatment of JE virus not only resulted in the irreversible loss of its HA activity but also affected the antigenic reactivity of HI epitopes on its **E** protein molecule.

L37 ANSWER 37 OF 83 MEDLINE on STN

96282646. PubMed ID: 8683218. Protective immune responses to the **E** and NS1 proteins of Murray Valley encephalitis virus in hybrids of **flavivirus**-resistant mice. Hall R A; Brand T N; Lobigs M; Sangster M Y; Howard M J; Mackenzie J S. (Department of Microbiology, University of Western Australia, Nedlands, Australia. ) The Journal of general virology, (1996 Jun) Vol. 77 ( Pt 6), pp. 1287-94. Journal code: 0077340. ISSN: 0022-1317. Pub. country: ENGLAND: United Kingdom. Language: English.

AB The lack of an effective animal model has been a major obstacle in attempts to define the role of humoral and cellular immune responses in protection against **flavivirus** infection. We have used F1 hybrid mice (BALB/c x C3H/RV) that are heterozygous for the **flavivirus** resistance allele Flvr and show reduced virus replication in the brain after intracerebral inoculation. F1 hybrid mice challenged by intracerebral inoculation with Murray Valley encephalitis (MVE) virus developed encephalitis 2-3 days later than a genetically susceptible strain (BALB/c) but showed a similar mortality rate. This delay in the onset of disease provided more opportunity for virus clearance by primed immune responses. Using F1 hybrid mice we were able to demonstrate protective immunity induced by structural and non-structural proteins of MVE virus by immunization with pure NS1 protein or recombinant vaccinia viruses that expressed various regions of the MVE genome. These constructs included VV-STR (C-**prM-E**-NS1-NS2A), VV-delta C (**prM-E**-NS1-NS2A) and VV-NS1 (NS1-NS2A). VV-delta C vaccinated mice were completely protected (100% survival) from challenge with 1000 infectious units of MVE virus, while mice inoculated with VV-STR, VV-NS1 or pure NS1 were partially protected (40%, 47% and 85% respectively). Analysis of prechallenge sera and in vivo depletion studies revealed that the solid protection induced by VV-delta C was mediated by neutralizing antibody to the **E** protein and did not require a CD8+ T cell response. The partial protection provided by VV-STR, VV-NS1 and pure NS1 occurred after induction of antibody to NS1. However, depletion of CD8+ cells prior to virus challenge ablated the protection provided by VV-NS1 indicating some requirement for class I restricted cytotoxic T cells.

L37 ANSWER 38 OF 83 MEDLINE on STN

96215657. PubMed ID: 8645110. Immunisation with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. Phillpotts R J; Venugopal K; Brooks T. (Microbiology Group, Chemical and Biological Defence Establishment, Porton Down, Wiltshire, U.K. ) Archives of virology, (1996) Vol. 141, No. 3-4, pp. 743-9. Journal code: 7506870. ISSN: 0304-8608. Pub. country: Austria. Language: English.

AB In vivo transfection by intramuscular injection with plasmids expressing the immunogenic proteins of microbial pathogens has considerable potential as a vaccination strategy against many pathogens of both man and animals. Here we report that weanling mice given a single intramuscular injection of 50 micrograms of a plasmid, pSLE1 expressing the St. Louis encephalitis virus (SLE) **prM/E** protein under the control of the cytomegalovirus immediate early protein promoter produced SLE-specific antibody and were protected against lethal challenge with the virulent virus. Polynucleotide vaccine technology provides a unique opportunity to produce vaccines against **flavivirus** diseases of low incidence cheaply and rapidly, and to produce multivalent vaccines such as would be required for immunisation against **dengue** virus disease.

L37 ANSWER 39 OF 83 MEDLINE on STN

96088052. PubMed ID: 8525682. Immunity to St. Louis encephalitis virus by sequential immunization with recombinant vaccinia and baculovirus derived **PrM/E** proteins. Venugopal K; Jiang W R; Gould E A. (Institute of Virology and Environmental Microbiology, Oxford, UK. ) Vaccine, (1995 Aug) Vol. 13, No. 11, pp. 1000-5. Journal code: 8406899. ISSN: 0264-410X. Pub. country: ENGLAND: United Kingdom. Language: English.

AB St. Louis encephalitis (SLE) is an important mosquito-borne disease of great public health concern in parts of the United States. South America and Canada. Protective immunogens of **flaviviruses** produced in different expression systems have been shown to be effective against virulent virus infection in laboratory animal models. Here we show that the pre-membrane and envelope (**PrM/E**) of SLE virus expressed in insect and mammalian cell systems using baculovirus and vaccinia virus, respectively, are processed correctly and showed similar antigenic characteristics as the authentic proteins. Immunization with the recombinant proteins individually or in combination resulted in neutralizing and protective immune responses. A schedule consisting of initial immunization with recombinant vaccinia virus followed by a secondary boost with recombinant baculovirus protein resulted in higher levels of neutralizing and protective immune responses. The advantages of the use of such a combined approach as a general immunization strategy are discussed.

L37 ANSWER 40 OF 83 MEDLINE on STN

96010231. PubMed ID: 7571428. Double-subgenomic Sindbis virus recombinants expressing immunogenic proteins of **Japanese encephalitis virus** induce significant protection in mice against lethal JEV infection.



AB A series of double-subgenomic Sindbis virus (dsSIN) recombinants that express cassettes encoding the immunogenic proteins of **Japanese encephalitis virus (JEV)** [**prM-E**, **prM-E-NS1**, **NS1-NS2A**, **80%E** (encodes the amino-terminal 80% part of **E**), and **NS1**] were constructed and analyzed for their ability to confer protective immunity in mice against lethal challenge with neurovirulent JEV. The cassettes were introduced into both 5' (second subgenomic promoter of the vector precedes the SIN structural open reading frame (SP-ORF)) and 3' (the promoter follows the SP-ORF) dsSIN vectors. The longest cassette (**prM-E-NS1**) was 3.2 kb in length, which is remarkable for such a small vector virus as SIN (SIN genome is roughly 11.8 kb in length). The level of expression of JEV proteins appeared similar for both 5' and 3' recombinants. In general, the stability of the recombinants obtained was found to be low (expression was lost following one to five passages at low multiplicity of infection, depending on the recombinant). However, 5' recombinants containing longer cassettes (**prM-E-NS1**, **prM-E**, **NS1-NS2A**) were more stable than the corresponding 3' recombinants. Intraperitoneal inoculation of mice with 10(7) PFU of dsSIN-JEV recombinants induced antibodies against JEV proteins and low titers of JEV-neutralizing antibodies were produced by mice inoculated with recombinants expressing **80%E**, **prM-E**, and **prM-E-NS1**. A single immunization of mice with the dsSIN-**prM-E** or dsSIN-**prM-E-NS1** recombinants provided 40-65% protection against peripheral lethal challenge with 10(4) LD50 of neurovirulent JEV. The results demonstrate that genetically engineered togaviruses can be successfully used as vaccine vectors.

L37 ANSWER 41 OF 83 MEDLINE on STN

95225741. PubMed ID: 7535997. Tick-borne encephalitis virus envelope protein **E**-specific monoclonal antibodies for the study of low pH-induced conformational changes and immature virions. Holzmann H; Stiasny K; York H; Dorner F; Kunz C; Heinz F X. (Institute of Virology, University of Vienna, Austria. ) Archives of virology, (1995) Vol. 140, No. 2, pp. 213-21. Journal code: 7506870. ISSN: 0304-8608. Pub. country: Austria. Language: English.

AB A set of ten monoclonal antibodies (mabs) specific for the tick-borne encephalitis (TBE) virus envelope protein **E** were prepared and characterized with respect to their functional activities, the location of their binding sites on protein **E** and the involvement of their epitopes in acid pH-induced conformational changes and interactions with the precursor to the membrane protein (**prM**) in immature virions. The majority of these mabs mapped to the previously defined antigenic domain A. All of the mabs recognize parts of the **E** protein which undergo low pH-induced structural rearrangements believed to be necessary for the fusion activity of the virus, and six of the mabs define epitopes which are affected by the **prM-E** interaction in immature virions. They are therefore of potential value as specific reagents for studying the structure and function of protein **E**, as well as the function of the **prM-E** association. Five of the mabs exhibited neutralizing activity, and can therefore be used for the selection of escape mutants.

L37 ANSWER 42 OF 83 MEDLINE on STN

95213156. PubMed ID: 7698880. Comparative analysis of NS3 sequences of temporally separated **dengue 3** virus strains isolated from southeast Asia. Chow V T; Seah C L; Chan Y C. (Department of Microbiology, Faculty of Medicine, National University of Singapore, Kent Ridge. ) Intervirology, (1994) Vol. 37, No. 5, pp. 252-8. Journal code: 0364265. ISSN: 0300-5526. Pub. country: Switzerland. Language: English.

AB By a combination of PCR and direct-cycle sequencing using consensus primers, we analyzed approximately 400-bp fragments within the NS3 genes of twenty-one **dengue** virus type 3 strains isolated from five neighboring Southeast Asian countries at different time intervals from 1956 to 1992. The majority of base disparities were silent mutations, with few predicted amino acid substitutions, thus emphasizing the strict conservation of the NS3 gene. Phylogenetic trees constructed on the basis of these nucleotide differences revealed distinct but related clusters of strains from the Philippines, Indonesia, and strains from Singapore and Malaysia of the 1970s and early 1980s, while the Thai cluster was relatively more distant. This genetic relationship was compatible with that proposed by other workers who have studied other **dengue 3** virus genes such as **E**, **M** and **prM**. However, we observed that the more recent, epidemic-associated **dengue 3** strains from Singapore and Malaysia of the late 1980s and early 1990s were more closely related to the Thai cluster, implying their evolution from the latter, and emphasizing the importance of viral spread via increasing travel within the Southeast Asian area and elsewhere. Nucleotide sequence analysis of the NS3 genes of **dengue** viruses can serve to advance the understanding of the epidemiology and evolution of these viruses.

L37 ANSWER 43 OF 83 MEDLINE on STN

of the viral NS2B-NS3 protease. Yamshchikov V F; Compans R W. (Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322. ) Journal of virology, (1995 Apr) Vol. 69, No. 4, pp. 1995-2003. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB One of the late processing events in the **flavivirus** replication cycle involves cleavage of the intracellular form of the **flavivirus** capsid protein (Cint) to the mature virion form (Cvir) lacking the carboxy-terminal stretch of hydrophobic amino acids which serves as a signal peptide for the downstream **prM** protein. This cleavage event was hypothesized to be effected by a viral protease and to be associated with virion formation. We have proposed a model of **flavivirus** virion formation in which processing of the C-**prM** precursor at the upstream signalase site is upregulated by interaction of the NS2B part of the protease with the **prM** signal peptide or with an adjacent carboxy-terminal region of the capsid protein in the precursor, and processing of Cint by the NS2B-NS3 protease follows the signalase cleavage. Recently, an alternative hypothesis was proposed which suggests a reverse order of these two cleavage events, namely, that cleavage of the C-**prM** precursor by the NS2B-NS3 protease at the Cint-->Cvir dibasic cleavage site is a prerequisite for the subsequent signalase cleavage of the **prM** signal peptide. To distinguish between these alternative models, we prepared a series of expression cassettes carrying mutations at the Cint-->Cvir dibasic cleavage site and investigated the effects of these mutations on signalase processing of C-**prM** and on formation and secretion of **prM-E** heterodimers. For certain mutated C-**prM** precursors, namely, for those with Lys-->Gly disruption of the dibasic site, efficient formation of **prM** was observed upon expression from larger cassettes encoding the viral protease, despite the absence of processing at the Cint-->Cvir cleavage site. Surprisingly, formation and secretion of **prM-E** heterodimers accompanied by late cleavage of **prM** was also observed for these cassettes, with an efficiency comparable to that of the wild-type expression cassette. These observations contradict the model in which cleavage of the C-**prM** precursor at the Cint-->Cvir dibasic site is a prerequisite for signalase cleavage.

L37 ANSWER 44 OF 83 MEDLINE on STN

95115117. PubMed ID: 7529335. Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. Allison S L; Schlich J; Stiasny K; Mandl C W; Kunz C; Heinz F X. (Institute of Virology, University of Vienna, Austria. ) Journal of virology, (1995 Feb) Vol. 69, No. 2, pp. 695-700. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB The **flavivirus** envelope protein **E** undergoes irreversible conformational changes at a mildly acidic pH which are believed to be necessary for membrane fusion in endosomes. In this study we used a combination of chemical cross-linking and sedimentation analysis to show that the envelope proteins of the **flavivirus** tick-borne encephalitis virus also change their oligomeric structure when exposed to a mildly acidic environment. Under neutral or slightly alkaline conditions, protein **E** on the surface of native virions exists as a homodimer which can be isolated by solubilization with the nonionic detergent Triton X-100. Solubilization with the same detergent after pretreatment at an acidic pH, however, yielded homotrimers rather than homodimers, suggesting that exposure to an acidic pH had induced a simultaneous weakening of dimeric contacts and a strengthening of trimeric ones. The pH threshold for the dimer-to-trimer transition was found to be 6.5. Because the pH dependence of this transition parallels that of previously observed changes in the conformation and hydrophobicity of protein **E** and that of virus-induced membrane fusion, it appears likely that the mechanism of fusion with endosomal membranes involves a specific rearrangement of the proteins in the viral envelope. Immature virions in which protein **E** is associated with the uncleaved precursor (**prM**) of the membrane protein **M** did not undergo a low-pH-induced rearrangement. This is consistent with a protective role of protein **prM** for protein **E** during intracellular transport of immature virions through acidic compartments of the trans-Golgi network.

L37 ANSWER 45 OF 83 MEDLINE on STN

95065714. PubMed ID: 7975266. Expression of cloned envelope protein genes from the **flavivirus** tick-borne encephalitis virus in mammalian cells and random mutagenesis by PCR. Allison S L; Mandl C W; Kunz C; Heinz F X. (Institute of Virology, University of Vienna, Austria. ) Virus genes, (1994 Jul) Vol. 8, No. 3, pp. 187-98. Journal code: 8803967. ISSN: 0920-8569. Pub. country: United States. Language: English.

AB The structural membrane proteins **prM** and **E** of the **flavivirus** tick-borne encephalitis (TBE) virus were expressed in mammalian cells for the purpose of probing the structure and molecular interactions of these proteins. Advantage was taken of the natural error frequency of the Taq polymerase used in the PCR amplification to generate a randomly mutated population of genes that were then cloned directly into plasmid expression vectors under the control of an SV40 promoter. Analysis of the mutation

produced mutations at a rate yielding an average of one to two amino acid changes per clone in the 496 amino acid long protein **E**. This is an ideal rate for assessing the importance of individual amino acid residues within protein domains, thus demonstrating the potential value of the PCR as a random mutagenesis method. Clones encoding wild-type **prM** and **E** proteins, and a truncated form of **E**, were also constructed by recombining portions of selected PCR clones. Transfection of COS-1 cells with these constructs resulted in expression of the **prM** and **E** proteins, which was demonstrated by indirect immunofluorescence using monoclonal antibodies (Mabs). The intracellular level of TBE virus antigen, measured in lysates of transfected cells by ELISA, reached approximately 25% of that found in virus-infected COS cells. Furthermore, it was shown by immunofluorescence using a panel of 19 anti-**E** Mabs that the antigenic structure of the expressed **E** proteins was nearly identical to that of **E** protein in infected cells, thus confirming the suitability of this model system as a tool for studying **flavivirus** protein structure.

L37 ANSWER 46 OF 83 MEDLINE on STN

95027681. PubMed ID: 7941319. Processing of **flavivirus** structural glycoproteins: stable membrane insertion of premembrane requires the envelope signal peptide. Markoff L; Chang A; Falgout B. (Laboratory of Vector-borne Virus Diseases, Food and Drug Administration, Bethesda, Maryland 20892. ) *Virology*, (1994 Nov 1) Vol. 204, No. 2, pp. 526-40. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB The **flavivirus** structural proteins capsid (C), premembrane (**prM**), and envelope (**E**) are cleaved in that order from the N-terminus of the polyprotein by the ER intraluminal enzyme signal peptidase. The **prM-E** and **E-NS1** junctions contain hydrophobic domains with both transmembrane and signal function. These domains reside at the C-termini of **prM** and **E**, respectively, after cleavage. We studied the functions of the 37-amino-acid C-terminus of the **dengue** virus type 4 (DEN4) **prM** (amino acids 243-279 of the DEN4 polyprotein) in the processing of **prM** and **E**. Hydrophobicity in this domain is interrupted by a conserved Arg residue (Arg-264) within a short amphipathic segment. Hydrophobic amino acids upstream from Arg-264 (aa 243-263) were presumed to constitute the membrane anchor for **prM** (the "tm" segment). Previous results had suggested that sequences downstream from Arg-264 (aa 265-279) constitute the **E** signal peptide. RNA transcripts prepared from wild-type (wt) and deletion-mutant DEN4 cDNAs encoding the **prM** signal peptide, **prM**, **E**, and the N-terminus of the nonstructural glycoprotein, NS1, were translated in rabbit reticulocyte lysate in the presence of microsomes. Processing of wt **prM** and **E** in vitro appeared to mimic processing occurring during **flavivirus** infection. Analysis of mutants confirmed the localization of the **E** signal peptide within residues 265 to 279. However, deletions within either the **E** signal peptide or the tm segment resulted in a defect in both membrane insertion of **prM** and cleavage of the **prM-E** junction. Membrane anchoring of **prM** appeared to be a two-step process requiring function of both the tm segment and the **E** signal peptide, and fully efficient **prM-E** cleavage was also dependent upon the integrity of both hydrophobic domains. We propose a model for the processing of the **flavivirus** structural glycoproteins based on these results.

L37 ANSWER 47 OF 83 MEDLINE on STN

94367626. PubMed ID: 8085382. Avipox virus-vectored **Japanese encephalitis virus** vaccines: use as vaccine candidates in combination with purified subunit immunogens. Konishi E; Pincus S; Paoletti E; Shope R E; Wason P W. (Department of Medical Zoology, Kobe University School of Medicine, Japan. ) *Vaccine*, (1994 May) Vol. 12, No. 7, pp. 633-8. Journal code: 8406899. ISSN: 0264-410X. Pub. country: ENGLAND: United Kingdom. Language: English.

AB An avipox virus, canarypox (ALVAC), which is naturally host-range restricted, was used to construct recombinants encoding the **Japanese encephalitis virus** (JEV) **prM**, **E** and NS1 genes (vCP107) and **prM** and **E** genes (vCP140). Mice immunized with these recombinant viruses produced JEV neutralizing antibodies and were protected from lethal JEV challenge. Protection was also observed in mice immunized with a subunit vaccine candidate, consisting of extracellular particles (EPs; RNA-free subviral membrane vesicles containing **prM/M** and **E** proteins) derived from HeLa cell cultures infected with a JEV-vaccinia recombinant. Mice primed with vCP107 and boosted with EPs had higher antibody levels than mice immunized twice with EPs alone, although the levels were comparable to that obtained in mice immunized twice with the recombinant virus. Mice immunized with a mixture of recombinant virus (vCP107) plus EPs had neutralizing antibody titres higher than mice immunized with the recombinant virus or EPs alone.

L37 ANSWER 48 OF 83 MEDLINE on STN

94305413. PubMed ID: 7913359. The interactions of the **flavivirus** envelope proteins: implications for virus entry and release. Heinz F X; Auer G; Stiasny K; Holzmann H; Mandl C; Guirakhoo F; Kunz C. (Institute of Virology, University of Vienna, Austria. ) *Archives of virology*.

0939-1983. Pub. country: Austria. Language: English.

- AB Viral membrane proteins play an important role in the assembly and disassembly of enveloped viruses. Oligomerization and proteolytic cleavage events are involved in controlling the functions of these proteins during virus entry and release. Using tick-borne encephalitis virus as a model we have studied the role of the **flavivirus** envelope proteins **E** and **prM**/M in these processes. Experiments with acidotropic agents provide evidence that the virus is taken up by receptor-mediated endocytosis and that the acidic pH in endosomes plays an important role for virus entry. The envelope glycoprotein **E** undergoes irreversible conformational changes at acidic pH, as indicated by the loss of several monoclonal antibody-defined epitopes, which coincide with the viral fusion activity in vitro. Sedimentation analysis reveals that these conformational changes lead to aggregation of virus particles, apparently by the exposure of hydrophobic sequence elements. None of these features are exhibited by immature virions containing **E** and **prM** rather than **E** and M. Detergent solubilization, sedimentation, and crosslinking experiments provide evidence that **prM** forms a complex with protein **E** which prevents the conformational changes necessary for fusion activity. The functional role of **prM** before its endoproteolytic cleavage by a cellular protease thus seems to be the protection of protein **E** from acid-inactivation during its passage through acidic trans Golgi vesicles in the course of virus release.

L37 ANSWER 49 OF 83 MEDLINE on STN

94219172. PubMed ID: 8165861. Recombinant vaccinia viruses co-expressing **dengue**-1 glycoproteins **prM** and **E** induce neutralizing antibodies in mice. Fonseca B A; Pincus S; Shope R E; Paoletti E; Mason P W. (Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT 06510. ) Vaccine, (1994) Vol. 12, No. 3, pp. 279-85. Journal code: 8406899. ISSN: 0264-410X. Pub. country: ENGLAND: United Kingdom. Language: English.

- AB Four recombinant vaccinia viruses expressing different portions of the **dengue** type 1 virus (DEN-1) genome (C-**prM-E**-NS1-NS2A-NS2B; **prM-E**; **prM-E**-NS1-NS2A-NS2B; or NS1-NS2A) were constructed in order to establish the most immunogenic configuration of DEN-1 proteins. Both recombinants producing **prM** and **E** in the absence of C induced the synthesis of extracellular forms of **E** in vitro. Mice inoculated with these two recombinants produced DEN-1 neutralizing (NEUT) and haemagglutination inhibiting (HAI) antibodies. The other two recombinant vaccinia viruses, which did not induce the production of extracellular forms of **E**, did not induce **E**-specific immune responses. These results support our previous studies on the design of **flavivirus**-vaccinia vaccine candidates by showing the importance of co-expressing **prM** and **E** in order to induce the synthesis of extracellular **E** and to elicit NEUT and HAI antibodies.

L37 ANSWER 50 OF 83 MEDLINE on STN

94082440. PubMed ID: 8259646. Structural changes and functional control of the tick-borne encephalitis virus glycoprotein **E** by the heterodimeric association with protein **prM**. Heinz F X; Stiasny K; Puschner-Auer G; Holzmann H; Allison S L; Mandl C W; Kunz C. (Institute of Virology, University of Vienna, Austria. ) Virology, (1994 Jan) Vol. 198, No. 1, pp. 109-17. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

- AB We have used tick-borne encephalitis virus to study the involvement of acidic compartments during the entry and release phases of **flavivirus** infection and to elucidate the role of protein **prM** in immature virions. Elevation of the pH in acidic intracellular compartments by either bafilomycin A1, a specific inhibitor of the vacuolar type H(+)-ATPase or by NH4Cl had a strong inhibitory effect during virus penetration and also prevented the cleavage of **prM** when added in the late phase of the viral life cycle. In the latter case the release of virus particles was not impaired. These immature (**prM**-containing) virions exhibited a 20- to 50-fold lower specific infectivity and HA activity than mature virions and in contrast to these did not undergo low pH-triggered aggregation. The presence of **prM** also affected the binding of monoclonal antibodies to protein **E**, especially at sites which have been shown to undergo acid pH-induced conformational changes in mature virions. Crosslinking, solubilization, and sedimentation analyses revealed the existence of **prM-E** heterooligomeric complexes, suggesting that the function of **prM** is to protect protein **E** from undergoing the irreversible conformational changes in acidic compartments of the secretory pathway that are necessary for triggering fusion activity in the endosome during virus entry.

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(FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006)

FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

L1 E CHANG GWONG J J/IN  
 2 S E4  
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 L2 24958 S CMV OR CMV-IE  
 L3 3918 S L2 AND KOZAK  
 L4 3136 S L3 AND TERMINATION  
 L5 0 S L4 AND (POLY W A)  
 L6 760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR  
 L7 33 S L6 AND CMV/CLM  
 L8 11 S L7 AND AY<1999  
 L9 498 S L6 AND PCDNA3?  
 L10 9 S L9 AND AY<1999  
 L11 3 S L10 NOT L8  
 E SCHMALJOHN C S/IN  
 L12 12 S E4  
 L13 4840 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELLOW FEVER  
 L14 161 S L13 AND (PRM? AND E)  
 L15 82 S L14 AND (SIGNAL SEQUENCE)  
 L16 15 S L15 AND AY<1999

FILE 'WPIDS' ENTERED AT 22:19:11 ON 01 JUN 2006

E CHANG G J/IN  
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 L18 3 S L17 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS O  
 L19 853 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRU OR YELLOW  
 L20 28 S L19 AND (PRM? AND E)  
 L21 4 S L20 AND CMV

FILE 'MEDLINE' ENTERED AT 22:28:38 ON 01 JUN 2006

E CHANG G J J/AU  
 L22 50 S E2  
 L23 23 S L22 AND (FLAVIVIR? OR DENGUE OR YELLOW FEVER OR JAPANESE ENCE  
 L24 16 S L23 AND PY<2000  
 E KONISHI E/AU  
 L25 17 S E6  
 L26 6 S E5  
 E KONISHI EIJI/AU  
 L27 25 S E2-E4  
 L28 16 S L27 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELL  
 L29 1144 S (JEV OR JAPANESE ENCEPHALITIS VIRUS)  
 L30 6 S L29 AND (SUBVIRAL PARTICLES)  
 L31 9634 S (FLAVIVIR? OR YELLOW FEVER VIRUS? OR JAPANESE ENCEPHALITIS VI  
 L32 1243 S L31 AND (PRM? OR E)  
 L33 233 S L32 AND (PRM?)  
 L34 194 S L33 AND (PRM AND E)  
 L35 88 S L34 AND PY<2000  
 L36 5 S L35 AND (SUBVIRAL PARTICLE? OR VIRUS-LIKE PARTICLE?)  
 L37 83 S L35 NOT L36

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L37 ANSWER 1 OF 83 MEDLINE on STN

2000337405. PubMed ID: 10879241. Antibody responses of **dengue** fever  
 patients to **dengue** 2 (New Guinea C strain) viral proteins. AbuBakar S;  
 Azmi A; Mohamed-Saad N; Shafee N; Chee H Y. (Department of Medical  
 Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur. )  
 The Malaysian journal of pathology, (1997 Jun) Vol. 19, No. 1, pp.  
 41-51. Journal code: 8101177. ISSN: 0126-8635. Pub. country: Malaysia.  
 Language: English.

AB The present study was undertaken to investigate the antibody responses of  
**dengue** fever (DF) patients to specific **dengue** virus proteins.  
 Partially purified **dengue** 2 New Guinea C (NGC) strain virus was used as  
 antigen. Under the present experimental protocols, it was observed that  
 almost all DF patients' sera had detectable presence of antibodies which  
 recognize the **dengue** 2 envelope (E) protein. The convalescent-phase  
 sera especially had significant detectable IgG, IgM and IgE against the  
 protein. In addition, IgGs specific against the NS1 dimer and PrM were  
 also detected. Antibody against the core (C) protein, however, was not  
 detectable in any of the DF patients' sera. The substantial presence of  
 IgG against the PrM in the convalescent-phase sera, and the presence of  
 IgE specific for the E, reflect the potential importance of these  
 antibody responses in the pathogenesis of **dengue**.

L37 ANSWER 2 OF 83 MEDLINE on STN

2000129485. PubMed ID: 10664386. Identification of an epitope on the  
**dengue** virus membrane (M) protein defined by cross-protective monoclonal  
 antibodies: design of an improved epitope sequence based on common  
 determinants present in both envelope (E and M) proteins. Falconar A K.  
 (Department of Infectious and Tropical Diseases, London School of Hygiene  
 and Tropical Medicine, London, U.K. ) Archives of virology, (1999) Vol.

country: Austria. Language: English.

- AB The protective capacity of monoclonal antibodies (MAbs) generated to the **dengue-2** virus envelope (**E**) and premembrane (**prM**) proteins was tested in vivo. Two anti-**E** MAbs, 2C5.1 and 4G2 and two anti-**prM** MAbs, 2A4.1 and 2H2 provided cross-protection against all four **dengue** virus serotypes. Overlapping sets of synthetic peptides spanning amino-acid sequence 301-401 (domain III) of the **E** protein and the entire **prM** protein were then used to locate their epitopes. The anti-**E** MAbs strongly reacted with the peptide sequence 349-GRLITVNPVIT-359 (E349-359) from domain III and the immunodominant epitope, 274-SGNLLFTGHL-283 (E274-283) from the hinge region between domains I and II. The anti-**prM** MAbs strongly reacted with the sequence, 40-PGFTVMAAIL-49 (M40-49) from the first membrane-spanning domain of the M protein. These anti-**prM** MAbs also reacted with peptides E274-283 and E349-359, while the anti-**E** MAbs reacted with a peptide sequence, 1-FHLTTRNGEP-10 from the **prM** protein and these cross-reactions with both proteins were confirmed using immunoblot assays. MAbs 2C5.1, 4G2 and 2H2 more strongly reacted with an MEH1 peptide GLFTPNLITI, which was designed as an antigenic hybrid between these **E** and **prM** peptide sequences, than with any of these natural peptide sequences. These peptide sequence will now be tested for their ability to generate cross-protective antibodies against each **dengue** virus serotype when delivered with appropriate T-helper epitopes.

L37 ANSWER 3 OF 83 MEDLINE on STN

2000013318. PubMed ID: 10544091. Evaluation of tick-borne encephalitis DNA vaccines in monkeys. Schmaljohn C; Custer D; VanderZanden L; Spik K; Rossi C; Bray M. (Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland 21702, USA.. connie.schmaljohn@amedd.army.mil) . Virology, (1999 Oct 10) Vol. 263, No. 1, pp. 166-74. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

- AB Tick-borne encephalitis is usually caused by infection with one of two **flaviviruses**: Russian spring summer encephalitis virus (RSSEV) or Central European encephalitis virus (CEEV). We previously demonstrated that gene gun inoculation of mice with naked DNA vaccines expressing the **prM** and **E** genes of these viruses resulted in long-lived homologous and heterologous protective immunity (Schmaljohn et al., 1997). To further evaluate these vaccines, we inoculated rhesus macaques by gene gun with the RSSEV or CEEV vaccines or with both DNA vaccines and compared resulting antibody titers with those obtained by vaccination with a commercial, formalin-inactivated vaccine administered at the human dose. Vaccinations were given at days 0, 30, and 70. All of the vaccines elicited antibodies detected by ELISA and by plaque-reduction neutralization tests. The neutralizing antibody responses persisted for at least 15 weeks after the final vaccination. Because monkeys are not uniformly susceptible to tick-borne encephalitis, the protective properties of the vaccines were assessed by passive transfer of monkey sera to mice and subsequent challenge of the mice with RSSEV or CEEV. One hour after transfer, mice that received 50 microl of sera from monkeys vaccinated with both DNA vaccines had circulating neutralizing antibody levels <20-80. All of these mice were protected from challenge with RSSEV or CEEV. Mice that received 10 microl of sera from monkeys vaccinated with the individual DNA vaccines, both DNA vaccines, or a commercial vaccine were partially to completely protected from RSSEV or CEEV challenge. These data suggest that DNA vaccines may offer protective immunity to primates similar to that obtained with a commercial inactivated-virus vaccine.  
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L37 ANSWER 4 OF 83 MEDLINE on STN

1999292853. PubMed ID: 10364309. Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein **E**. Allison S L; Stiasny K; Stadler K; Mandl C W; Heinz F X. (Institute of Virology, University of Vienna, Vienna, Austria.. steven.allison@univie.ac.at) . Journal of virology, (1999 Jul) Vol. 73, No. 7, pp. 5605-12. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

- AB Envelope protein **E** of the **flavivirus** tick-borne encephalitis virus mediates membrane fusion, and the structure of the N-terminal 80% of this 496-amino-acid-long protein has been shown to differ significantly from that of other viral fusion proteins. The structure of the carboxy-terminal 20%, the stem-anchor region, is not known. It contains sequences that are important for membrane anchoring, interactions with **prM** (the precursor of membrane protein M) during virion assembly, and low-pH-induced structural changes associated with the fusion process. To identify specific functional elements in this region, a series of C-terminal deletion mutants were constructed and the properties of the resulting truncated recombinant **E** proteins were examined. Full-length **E** proteins and proteins lacking the second of two predicted transmembrane segments were secreted in a particulate form when coexpressed with **prM**, whereas deletion of both segments resulted in the secretion of soluble homodimeric **E** proteins. Sites located within a

the first membrane-spanning region (amino acids 450 to 472) were found to be important for the stability of the **prM-E** heterodimer but not essential for **prM**-mediated intracellular transport and secretion of soluble **E** proteins. A separate site in the stem, also corresponding to a predicted alpha-helix (amino acids 401 to 413), was essential for the conversion of soluble protein **E** dimers to a homotrimeric form upon low-pH treatment, a process resembling the transition to the fusogenic state in whole virions. This functional mapping will aid in the understanding of the molecular mechanisms of membrane fusion and virus assembly.

L37 ANSWER 5 OF 83 MEDLINE on STN

1999292845. PubMed ID: 10364301. The anamnestic neutralizing antibody response is critical for protection of mice from challenge following vaccination with a plasmid encoding the **Japanese encephalitis virus** premembrane and envelope genes. Konishi E; Yamaoka M; Khin-Sane-Win; Kurane I; Takada K; Mason P W. (Department of Health Sciences, Kobe University School of Medicine, Kobe 654-0142, Japan.. ekon@ams.kobe-u.ac.jp) . Journal of virology, (1999 Jul) Vol. 73, No. 7, pp. 5527-34. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB For Japanese encephalitis (JE), we previously reported that recombinant vaccine-induced protection from disease does not prevent challenge virus replication in mice. Moreover, DNA vaccines for JE can provide protection from high challenge doses in the absence of detectable prechallenge neutralizing antibodies. In the present study, we evaluated the role of postchallenge immune responses in determining the outcome of JE virus infection, using mice immunized with a plasmid, pcDNA3JEME, encoding the JE virus premembrane (**prM**) and envelope (**E**) coding regions. In the first experiment, 10 mice were vaccinated once (five animals) or twice (remainder) with 100 micrograms of pcDNA3JEME. All of these mice showed low (6 of 10) or undetectable (4 of 10) levels of neutralizing antibodies. Interestingly, eight of these animals showed a rapid rise in neutralizing antibody following challenge with 10,000 50% lethal doses of JE virus and survived for 21 days, whereas only one of the two remaining animals survived. No unimmunized animals exhibited a rise of neutralizing antibody or survived challenge. Levels of JE virus-specific immunoglobulin M class antibodies were elevated following challenge in half of the unimmunized mice and in the single pcDNA3JEME-immunized mouse that died. In the second experiment, JE virus-specific primary cytotoxic T-lymphocyte (CTL) activity was detected in BALB/c mice immunized once with 100 micrograms of pcDNA3JEME 4 days after challenge, indicating a strong postchallenge recall of CTLs. In the third experiment, evaluation of induction of CTLs and antibody activity by plasmids containing portions of the **prM/E** cassette demonstrated that induction of CTL responses alone were not sufficient to prevent death. Finally, we showed that antibody obtained from pcDNA3JEME-immunized mice 4 days following challenge could partially protect recipient mice from lethal challenge. Taken together, these results indicate that neutralizing antibody produced following challenge provides the critical protective component in pcDNA3JEME-vaccinated mice.

L37 ANSWER 6 OF 83 MEDLINE on STN

1999252164. PubMed ID: 10233934. **Dengue** virus structural differences that correlate with pathogenesis. Leitmeyer K C; Vaughn D W; Watts D M; Salas R; Villalobos I; de Chacon; Ramos C; Rico-Hesse R. (Department of Virology and Immunology, Southwest Foundation for Biomedical Research, San Antonio, Texas 78227-5301, USA. ) Journal of virology, (1999 Jun) Vol. 73, No. 6, pp. 4738-47. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB The understanding of **dengue** virus pathogenesis has been hampered by the lack of in vitro and in vivo models of disease. The study of viral factors involved in the production of severe **dengue**, **dengue** hemorrhagic fever (DHF), versus the more common **dengue** fever (DF), have been limited to indirect clinical and epidemiologic associations. In an effort to identify viral determinants of DHF, we have developed a method for comparing **dengue** type 2 genomes (reverse transcriptase PCR in six fragments) directly from patient plasma. Samples for comparison were selected from two previously described **dengue** type 2 genotypes which had been shown to be the cause of DF or DHF. When full genome sequences of 11 **dengue** viruses were analyzed, several structural differences were seen consistently between those associated with DF only and those with the potential to cause DHF: a total of six encoded amino acid charge differences were seen in the **prM**, **E**, NS4b, and NS5 genes, while sequence differences observed within the 5' nontranslated region (NTR) and 3' NTR were predicted to change RNA secondary structures. We hypothesize that the primary determinants of DHF reside in (i) amino acid 390 of the **E** protein, which purportedly alters virion binding to host cells; (ii) in the downstream loop (nucleotides 68 to 80) of the 5' NTR, which may be involved in translation initiation; and (iii) in the upstream 300 nucleotides of the 3' NTR, which may regulate viral replication via the formation of replicative intermediates. The significance of four amino

transport protein and the viral RNA polymerase, respectively, remains unknown. This new approach to the study of **dengue** virus genome differences should better reflect the true composition of viral RNA populations in the natural host and permit their association with pathogenesis.

L37 ANSWER 7 OF 83 MEDLINE on STN

1999231936. PubMed ID: 10217584. Recombinant, chimaeric live, attenuated vaccine (ChimeriVax) incorporating the envelope genes of Japanese encephalitis (SA14-14-2) virus and the capsid and nonstructural genes of yellow fever (17D) virus is safe, immunogenic and protective in non-human primates. Monath T P; Soike K; Levenbook I; Zhang Z X; Arroyo J; Delagrave S; Myers G; Barrett A D; Shope R E; Ratterree M; Chambers T J; Guirakhoo F. (OraVax Inc., Cambridge, MA 02139, USA.. tmonath@oravax.com) . Vaccine, (1999 Apr 9) Vol. 17, No. 15-16, pp. 1869-82. Journal code: 8406899. ISSN: 0264-410X. Pub. country: ENGLAND: United Kingdom. Language: English.

AB Yellow fever 17D virus, a safe and effective live, attenuated vaccine, was used as a vector for genes encoding the protective antigenic determinants of a heterologous member of the genus **Flavivirus**, Japanese encephalitis (JE) virus, the leading cause of acute viral central nervous system infection and death throughout Asia. The viral envelope (**prM** and **E**) genes of a full-length cDNA clone of YF 17D virus were replaced with the corresponding genes of JE SA14-14-2, a strain licensed as a live, attenuated vaccine in China. Full-length RNA transcripts of the YF/JE chimaera were used to transfect Vero cells. The progeny virus (named 'ChimeriVax-JE'), was used to define safety after intracerebral (i.c.) inoculation of rhesus monkeys. Monkeys (N = 3) inoculated with a high dose (6.6 log<sub>10</sub> pfu) developed a brief viremia, showed no signs of illness, developed high titers of anti-JE neutralizing antibody, and had minimal brain and spinal cord lesion scores according to criteria specified in the WHO monkey neurovirulence test. A control group of 3 monkeys that received a lower dose (4.2 log<sub>10</sub> pfu) of commercial YF 17D vaccine had slightly higher lesion scores. To develop a lethal monkey model of JE for vaccine protection tests, we inoculated groups of monkeys i.c. or intranasally (i.n.) with a JE virus strain found to be highly neurovirulent and neuroinvasive for mice. Monkeys inoculated i.c., but not i.n., developed severe encephalitis after an incubation period of 8-13 days. The ChimeriVax-JE virus was passed in a cell line acceptable for human use (diploid fetal rhesus lung) and 4.3 or 5.3 log<sub>10</sub> pfu were inoculated into groups of 3 monkeys by the subcutaneous route. All 6 animals developed brief viremias (peak titer < 2.0 log<sub>10</sub> pfu/ml) and subsequently had anti-JE but no yellow fever neutralizing antibodies. On day 64, the monkeys were challenged i.c. with 5.5 log<sub>10</sub> pfu of virulent JE virus. The immunized animals had no detectable viremia post-challenge, whereas 4 unimmunized controls became viremic. Only 1 of 6 (17%) vaccinated monkeys but 4 of 4 (100%) unvaccinated controls developed encephalitis. Histopathological examination 30 days after challenge confirmed that the protected, immunized animals had no or minimal evidence of encephalitis. These data demonstrated the ability of the ChimeriVax-JE to induce a rapid humoral immune response and to protect against a very severe, direct intracerebral virus challenge. Target areas of neuronal damage and inflammation in monkeys infected IC with wild-type JE, the chimaeric virus and YF 17D were similar, indicating that the histopathological scoring system used for the WHO yellow fever monkey neurovirulence test will be applicable to control testing of chimaeric seed viruses and vaccines.

L37 ANSWER 8 OF 83 MEDLINE on STN

1999174007. PubMed ID: 10074160. Yellow fever/Japanese encephalitis chimeric viruses: construction and biological properties. Chambers T J; Nestorowicz A; Mason P W; Rice C M. (Department of Molecular Microbiology and Immunology, St. Louis University Health Sciences Center, St. Louis, Missouri 63104, USA.. chambetj@wpogate.slu.edu) . Journal of virology, (1999 Apr) Vol. 73, No. 4, pp. 3095-101. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB A system has been developed for generating chimeric yellow fever/Japanese encephalitis (YF/JE) viruses from cDNA templates encoding the structural proteins **prM** and **E** of JE virus within the backbone of a molecular clone of the YF17D strain. Chimeric viruses incorporating the proteins of two JE strains, SA14-14-2 (human vaccine strain) and JE Nakayama (JE-N [virulent mouse brain-passaged strain]), were studied in cell culture and laboratory mice. The JE envelope protein (**E**) retained antigenic and biological properties when expressed with its **prM** protein together with the YF capsid; however, viable chimeric viruses incorporating the entire JE structural region (C-**prM-E**) could not be obtained. YF/JE(**prM-E**) chimeric viruses grew efficiently in cells of vertebrate or mosquito origin compared to the parental viruses. The YF/JE SA14-14-2 virus was unable to kill young adult mice by intracerebral challenge, even at doses of 10(6) PFU. In contrast, the YF/JE-N virus was neurovirulent, but the phenotype resembled parental YF virus rather than JE-N. Ten predicted amino acid differences distinguish the JE **E** proteins of the two chimeric viruses, therefore implicating one or more residues as



system. This study indicates the feasibility of expressing protective antigens of JE virus in the context of a live, attenuated **flavivirus** vaccine strain (YF17D) and also establishes a genetic system for investigating the molecular basis for neurovirulence determinants encoded within the JE **E** protein.

L37 ANSWER 9 OF 83 MEDLINE on STN

1999145404. PubMed ID: 10022806. Retrospective study of Western blot profiles in immune sera of natural **dengue** virus infections. Se-Thoe S Y; Ng M M; Ling A E. (Department of Microbiology, National University of Singapore, Singapore General Hospital, Republic of Singapore. ) Journal of medical virology, (1999 Mar) Vol. 57, No. 3, pp. 322-30. Journal code: 7705876. ISSN: 0146-6615. Pub. country: United States. Language: English.

AB The Western blot (WB) assay was used to determine **dengue** virus antibodies present in human immune sera arising from recent primary and secondary **dengue** virus infections in Singapore. Cell lysates of **dengue**-2 virus-infected C6/36 and Vero cells were used. Antibodies directed against structural proteins of **dengue**-2 virus including envelope (**E**, gp60/50), capsid-premembrane (C-**PrM**, gp35), and premembrane (**PrM**, gp20) were detected, with antibody against envelope protein being most dominant. Similar WB profiles were detected in both primary and secondary **dengue** virus infections. The reactivity rate of antibodies to **dengue**-2 virus proteins was higher in infected Vero cell lysate than in infected C6/36 cell lysate, with the exception of antibodies to nonstructural proteins of NS1 and NS3, which were detected predominantly in infected C6/36 cell lysate. More than 75% of "normal" individuals (with no complaint of recent **dengue** virus infection) examined had low levels of **dengue** virus antibodies, but all presented with similar WB profiles as patients with recent **dengue** virus infections. This finding reflects a high seroprevalence of **dengue** virus infections and the long lasting nature of **E**, C-**PrM**, and **PrM** antibodies. Results from this study indicate that in natural **dengue** virus infections, native **E**, C-**PrM**, and **PrM** antigens of **dengue** virus are immunogenic and elicit long-lasting antibodies.

L37 ANSWER 10 OF 83 MEDLINE on STN

1999141635. PubMed ID: 9987162. Protection against lethal **Japanese encephalitis virus** infection of mice by immunization with the highly attenuated MVA strain of vaccinia virus expressing JEV **prM** and **E** genes. Nam J H; Wyatt L S; Chae S L; Cho H W; Park Y K; Moss B. (Department of Viral Disease, Korean NIH, Seoul, Korea. ) Vaccine, (1999 Jan 21) Vol. 17, No. 3, pp. 261-8. Journal code: 8406899. ISSN: 0264-410X. Pub. country: ENGLAND: United Kingdom. Language: English.

AB Genes encoding the glycosylated precursor of the membrane (**prM**) and envelope (**E**) proteins of a Korean strain of **Japanese encephalitis virus** (JEV) were inserted into the genome of the host-range restricted, highly attenuated, and safety-tested MVA strain of vaccinia virus. MVA recombinants containing the JEV genes, under strong synthetic or modified H5 vaccinia virus promoters, were isolated. Synthesis of JEV **prM** and **E** proteins was detected by immunofluorescence microscopy, flow cytometry, and polyacrylamide gel electrophoresis. Mice inoculated and boosted by various routes with either of the MVA recombinants produced JEV neutralizing antibodies, that had titres comparable with those induced by an inactivated JEV vaccine, as well as haemagglutination-inhibiting antibodies. Mice immunized with  $2 \times 10^6$  infectious units of MVA/JEV recombinants by intramuscular or intraperitoneal routes were completely protected against a  $10^5$  LD50 JEV challenge at 9 weeks of age.

L37 ANSWER 11 OF 83 MEDLINE on STN

1999139041. PubMed ID: 9971841. **PrM**- and cell-binding domains of the **dengue** virus **E** protein. Wang S; He R; Anderson R. (Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada. ) Journal of virology, (1999 Mar) Vol. 73, No. 3, pp. 2547-51. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB The **E-prM** proteins of **flaviviruses** are unusual complexes which play important roles in virus assembly and fusion modulation and in potential immunity-inducing vaccines. Despite their importance, little is known about the biogenesis and structural organization of **E-prM** complexes. Pulse-chase radiolabeling of **dengue** virus-infected Vero cells demonstrated a rapid interassociation of **E** and **prM** proteins, and sucrose gradient sedimentation analysis suggested that **E-prM** complexes progressed from simple heteromers to more densely sedimenting structures indicating increased multimerization. **E-prM** heteromers of even higher complexity were observed in virus particles, suggesting an intracellular assembly process which results in the networking of **E-prM** subunits into a lattice-like structure found in virus particles. Trypsin cleavage of **E-prM**-containing virus particles resulted in the release of a soluble 45-kDa fragment of the **E** protein which retained cell-binding activity. The results suggest that **E-prM** interactions in **dengue** virus particles are largely mediated by domains in the carboxy-terminal anchoring domain of **E**, while cell-binding activity is retained in a

L37 ANSWER 12 OF 83 MEDLINE on STN

1999036024. PubMed ID: 9820138. Growth restriction of **dengue** virus type 2 by site-specific mutagenesis of virus-encoded glycoproteins. Pryor M J; Gualano R C; Lin B; Davidson A D; Wright P J. (Department of Microbiology, Monash University, Clayton, Victoria, Australia. ) The Journal of general virology, (1998 Nov) Vol. 79 ( Pt 11), pp. 2631-9. Journal code: 0077340. ISSN: 0022-1317. Pub. country: ENGLAND: United Kingdom. Language: English.

AB The three **flavivirus** glycoproteins **prM**, **E** and NS1 are formed by post-translational cleavage and are glycosylated by the addition of N-linked glycans. NS1 may form homodimers, whereas **E** may form homodimers, homotrimers or heterodimers (**prM-E**). Modification of these processes by mutagenesis of the proteins has the potential to generate viruses that are restricted in growth and are possible vaccine candidates. Using an SV40-based expression system, we previously analysed dimerization and secretion of the NS1 protein of **dengue** virus type 2 (DEN-2) with mutations in the conserved Cys residues, or within hydrophilic or hydrophobic regions, or at glycosylation sites. In this study, mutations which reduce cleavage at the DEN-2 **prM/E** signalase cleavage site are described. On the basis of earlier and current results with transient expression, six mutations which reduced NS1 dimerization and two mutations which inhibited **prM/E** cleavage were analysed individually for their effects on virus growth using a genomic length cDNA clone. Two viruses were obtained that showed reduced growth in cell culture and attenuation of neurovirulence when inoculated into 3-day-old mice. One of these viruses encoded NS1 that lacked the second glycosylation site, the other encoded a Ser --> Ile change at the -3 position of the **prM/E** cleavage site. A third virus encoding a mutation in NS1 within a hydrophilic region grew as well as the parental virus. No virus was detected for the remaining five mutations.

L37 ANSWER 13 OF 83 MEDLINE on STN

1998445455. PubMed ID: 9770429. DNA-based and alphavirus-vectored immunisation with **prM** and **E** proteins elicits long-lived and protective immunity against the **flavivirus**, Murray Valley encephalitis virus. Colombage G; Hall R; Pavy M; Lobigs M. (John Curtin School of Medical Research, The Australian National University, Canberra, ACT, 2601, Australia. ) Virology, (1998 Oct 10) Vol. 250, No. 1, pp. 151-63. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB The immunogenicity and protective efficacy of DNA-based vaccination with plasmids encoding the membrane proteins **prM** and **E** of the **flavivirus** Murray Valley encephalitis virus (MVE) were investigated. Gene gun-mediated intradermal delivery of DNA encoding the **prM** and **E** proteins elicited long-lived, virus-neutralising antibody responses in three inbred strains of mice and provided protection from challenge with a high titer inoculum of MVE. Intramuscular DNA vaccination by needle injection also induced MVE-specific antibodies that conferred resistance to challenge with live virus but failed to reduce virus infectivity in vitro. The two routes of DNA-based vaccination with **prM** and **E** encoding plasmids resulted in humoral immunity with distinct IgG subtypes. MVE-specific IgG1 antibodies were always prevalent after intradermal DNA vaccination via a gene gun but not detected when mice were immunised with DNA by the intramuscular route or infected with live virus. We also tested a Semliki Forest virus replicon as vector for a **flavivirus prM** and **E** protein-based subunit vaccine. Single-cycle infections in mice vaccinated with packaged recombinant replicon particles elicited durable, MVE-specific, and virus-neutralising antibody responses. Copyright 1998 Academic Press.

L37 ANSWER 14 OF 83 MEDLINE on STN

1998419473. PubMed ID: 9747336. Partial nucleotide sequence of **Japanese encephalitis virus** ling strain genome and comparison of the encoded structural proteins and nonstructural protein NS1 among **Japanese encephalitis virus** strains. Jan L R; Chen K L; Lu C F; Horng C B. (National Institute of Preventive Medicine, Department of Health, Taipei, Taiwan, R.O.C. ) Zhonghua Minguo wei sheng wu ji mian yi xue za zhi = Chinese journal of microbiology and immunology, (1994 May) Vol. 27, No. 2, pp. 80-9. Journal code: 8008067. ISSN: 0253-2662. Pub. country: TAIWAN: Taiwan, Province of China. Language: English.

AB Approximately 4000 nucleotides of the 5'-terminal portion of **Japanese encephalitis virus** (JEV) Ling strain genome were cloned and sequenced. This nucleotide sequence and its encoded C-**PrM-E**-NS1 polyprotein sequences were also compared with the corresponding sequences of other JE virus strains. Results demonstrated that the nucleotide sequence homology varied from 97.1 to 99.3% and the amino acid homology 98.6 to 98.9%. Based on homology, the Ling strain was closer to the Beijing-1 strain than to the SA14 and JaOArS982 strains. However, only on comparison of the **E** sequence, which neutralization, hemagglutination-inhibition and complement fixation antigenic determinants are located, between Ling and other JEV strains demonstrated that nucleotide sequence homology varied from 97.1%

is more closely related to the Beijing-1 strain than to the Nakayama NIH, SA14 and JaOArS982 strains in that order. Based on this analysis, the Taiwanese JEV strain appears to be more closely related to the Chinese strain than to the Japanese strain. Also, JEV strains isolated in humans are more closely related to each other than to JEV strains of mosquito isolates.

L37 ANSWER 15 OF 83 MEDLINE on STN

1998291412. PubMed ID: 9627942. Induction of **Japanese encephalitis virus**-specific cytotoxic T lymphocytes in humans by poxvirus-based JE vaccine candidates. Konishi E; Kurane I; Mason P W; Shope R E; Kanasa-Thanan N; Smuchny J J; Hoke C H Jr; Ennis F A. (Department of Medical Zoology, Kobe University School of Medicine, Japan.. ekon@ams.kobe-u.ac.jp) . Vaccine, (1998 May) Vol. 16, No. 8, pp. 842-9. Journal code: 8406899. ISSN: 0264-410X. Pub. country: ENGLAND: United Kingdom. Language: English.

AB Poxvirus-based recombinant Japanese encephalitis (JE) vaccine candidates, NYVAC-JEV and ALVAC-JEV, were examined for their ability to induce JE virus-specific cytotoxic T lymphocytes (CTLs) in a phase I clinical trial. These vaccine candidates encoded the JE virus premembrane (**prM**), envelope (**E**) and non-structural 1 (NS1) proteins. The volunteers received subcutaneous inoculations with each of these candidates on days 0 and 28, and blood was drawn 2 days before vaccination and on day 58. Anti-**E** and anti-NS1 antibodies were elicited in most vaccinees inoculated with NYVAC-JEV and in some vaccinees inoculated with ALVAC-JEV. Peripheral blood mononuclear cells (PBMCs) obtained from approximately one half of vaccinees showed positive proliferation in response to stimulation with live JE virus. Cytotoxic assays demonstrated the presence of JE virus-specific CTLs in in vitro-stimulated PBMCs obtained from two NYVAC-JEV and two ALVAC-JEV vaccinees. Cell depletion tests using PBMCs from one NYVAC-JEV recipient indicated that the phenotype of CTLs was CD8+CD4-.

L37 ANSWER 16 OF 83 MEDLINE on STN

1998285757. PubMed ID: 9621059. Encapsidation of the **flavivirus** kunjin replicon RNA by using a complementation system providing Kunjin virus structural proteins in trans. Khromykh A A; Varnavski A N; Westaway E G. (Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital, Brisbane, Queensland 4029, Australia.. A.Khromykh@mailbox.uq.edu.au) . Journal of virology, (1998 Jul) Vol. 72, No. 7, pp. 5967-77. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB Kunjin virus (KUN) replicon RNA was encapsidated by a procedure involving two consecutive electroporations of BHK-21 cells, first with KUN replicon RNA C20DXrep (with **prME** and most of C deleted) and about 24 h later with a recombinant Semliki Forest virus (SFV) replicon RNA(s) expressing KUN structural proteins. The presence of KUN replicon RNA in encapsidated particles was demonstrated by its amplification and expression in newly infected BHK-21 cells, detected by Northern blotting with a KUN-specific probe and by immunofluorescence analysis with anti-NS3 antibodies. No infectious particles were produced when C20DXrep RNA and recombinant SFV RNAs were electroporated simultaneously. When the second electroporation was performed with a single SFV replicon RNA expressing the KUN contiguous **prME** genes and the KUN C gene together but under control of two separate 26S subgenomic promoters (SFV-**prME**-C107), a 10-fold-higher titer of infectious particles was achieved than when two different SFV replicon RNAs expressing the KUN C gene (SFV-C107) and **prME** genes (SFV-**prME**) separately were used. No SFV replicon RNAs expressing KUN structural proteins were encapsidated in secreted particles. Infectious particles pelleted by ultracentrifugation of the culture fluid from cells sequentially transfected with C20DXrep and SFV-**prME**-C107 RNAs were neutralized by preincubation with monoclonal antibodies to KUN **E** protein. Radioimmunoprecipitation analysis with anti-**E** antibodies of the culture fluid of the doubly transfected cells showed the presence of C, **prM/M**, and **E** proteins in the immunoprecipitated particles. Reverse transcription-PCR analysis showed that the immunoprecipitated particles also contained KUN-specific RNA. The encapsidated replicon particles sedimented more slowly than KUN virions in a 5 to 25% sucrose density gradient and were uniformly spherical, with an approximately 35-nm diameter, compared with approximately 50 nm for KUN virions. The results of this study demonstrate for the first time packaging of **flavivirus** RNA in trans, and they exclude a role in packaging for virtually all of the structural region. Possible applications of the developed packaging system include the definition of the packaging signal(s) in **flavivirus** RNA as well as the amino acid motif(s) in the structural proteins involved in RNA encapsidation, virion assembly, and secretion. Furthermore, it could facilitate the development of a noninfectious vaccine delivery system based on encapsidation of a noncytopathic **flavivirus** replicon expressing heterologous genes.

L37 ANSWER 17 OF 83 MEDLINE on STN

1998241731. PubMed ID: 9573260. Induction of protective immunity against

**Japanese encephalitis virus** premembrane and envelope genes. Konishi E; Yamaoka M; Khin-Sane-Win; Kurane I; Mason P W. (Department of Medical Zoology, Kobe University School of Medicine, Kobe 650, Japan.. ekon@ams.kobe-u.ac.jp) . Journal of virology, (1998 Jun) Vol. 72, No. 6, pp. 4925-30. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

- AB A DNA vaccine plasmid containing the Japanese encephalitis (JE) virus premembrane (**prM**) and envelope (**E**) genes (designated pcDNA3JEME) was evaluated for immunogenicity and protective efficacy in mice. Two immunizations of 4-week-old female ICR mice with pcDNA3JEME by intramuscular or intradermal injections at a dose of 10 or 100 microg per mouse elicited neutralizing (NEUT) antibodies at titers of 1:10 to 1:20 (90% plaque reduction), and all immunized mice survived a challenge with 10,000 50% lethal doses of the P3 strain of JE virus. A single immunization with 100 microg of pcDNA3JEME did not elicit detectable NEUT antibodies but induced protective immunity. Spleen cells obtained from BALB/c mice immunized once with 10 or 100 microg of pcDNA3JEME contained JE virus-specific memory cytotoxic T lymphocytes (CTLs). BALB/c mice maintained detectable levels of memory B cells and CTLs for at least 6 months after one immunization with pcDNA3JEME at a dose of 100 microg. The CTLs induced in BALB/c mice immunized twice with 100 microg of pcDNA3JEME were CD8 positive and recognized mainly the envelope protein. These results indicate that pcDNA3JEME has the ability to induce a protective immune response which includes JE virus-specific antibodies and CTLs.

L37 ANSWER 18 OF 83 MEDLINE on STN

1998178636. PubMed ID: 9519821. Identification of a major determinant of mouse neurovirulence of **dengue** virus type 2 using stably cloned genomic-length cDNA. Gualano R C; Pryor M J; Cauchi M R; Wright P J; Davidson A D. (Department of Microbiology, Monash University, Clayton, Victoria, Australia. ) The Journal of general virology, (1998 Mar) Vol. 79 ( Pt 3), pp. 437-46. Journal code: 0077340. ISSN: 0022-1317. Pub. country: ENGLAND: United Kingdom. Language: English.

- AB A genomic-length cDNA clone corresponding to the RNA of **dengue** virus type 2 (DEN-2) New Guinea C strain (NGC) was constructed in a low copy number vector. The cloned cDNA was stably propagated in *Escherichia coli* and designated pDVWS501. RNA transcripts produced in vitro from the cDNA using T7 RNA polymerase yielded infectious virus (MON501) upon electroporation into BHK-21 cells. When compared with parental NGC virus, MON501 replicated to similar levels in *Aedes albopictus* C6/36 cells and showed similar neurovirulence in suckling mice. In contrast, a second genomic-length cDNA clone (pDVWS310) used as an intermediate in the construction of pDVWS501 produced virus (MON310) that replicated well in C6/36 cells but was not neurovirulent in mice. MON310 contained the **prM** and **E** genes of the non-neurovirulent PUO-218 strain in an NGC background. There were seven amino acid differences between the **prM** and **E** proteins of MON310 and MON501. The differences were generally conservative, with the exception of **E** residue 126, which was Glu in MON310 and Lys in MON501. To examine the role of this residue in mouse neurovirulence, substitutions of Glu --> Lys and Lys --> Glu were made in MON310 and MON501, respectively. The properties of these mutants clearly demonstrated that Lys at **E** residue 126 is a major determinant of DEN-2 mouse neurovirulence.

L37 ANSWER 19 OF 83 MEDLINE on STN

1998139149. PubMed ID: 9499109. Characterization of defective viral RNA produced during persistent infection of Vero cells with Murray Valley encephalitis virus. Lancaster M U; Hodgetts S I; Mackenzie J S; Urosevic N. (Department of Microbiology, University of Western Australia, Nedlands. ) Journal of virology, (1998 Mar) Vol. 72, No. 3, pp. 2474-82. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

- AB Defective interfering viral particles are readily produced in cell culture after a high multiplicity of infection with many animal RNA viruses. Due to defects that they carry in their genomes, their life cycle needs to be complemented by the helper functions provided by a parental virus which makes them both dependent on and competitive with the parental virus. In many instances, this may cause the abrogation of a lytic cycle of the parental virus, leading to a persistent infection. In this paper, we describe for the first time the presence of truncated or defective interfering viral RNAs produced in Vero cells persistently infected with the **flavivirus** Murray Valley encephalitis virus. While these RNAs have not been detected in acutely infected Vero cells, their appearance coincided with the establishment of persistent infection. We also show for the first time that the defective viral RNAs replicate well in both cell culture and cell-free virus replication systems, indicating that they may interfere with the replication of parental virus at the level of viral RNA synthesis. Significantly, structural analyses of these RNA species including nucleotide sequencing have revealed that they carry similar nucleotide deletions encompassing the genes coding for the **prM** and **E** proteins and various gene segments coding for the N terminus of the NS1

truncated NS1 proteins to occur in persistently infected cells. This may have further implications for the interference with the parental virus at the level of viral RNA synthesis in addition to a major one at the level of virion assembly and release.

L37 ANSWER 20 OF 83 MEDLINE on STN

1998080404. PubMed ID: 9420215. DNA immunization with **Japanese encephalitis virus** nonstructural protein NS1 elicits protective immunity in mice. Lin Y L; Chen L K; Liao C L; Yeh C T; Ma S H; Chen J L; Huang Y L; Chen S S; Chiang H Y. (Institute of Preventive Medicine, National Defense Medical Center, Taipei, Taiwan, Republic of China.. yll@msll.hinet.net) . Journal of virology, (1998 Jan) Vol. 72, No. 1, pp. 191-200. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB **Japanese encephalitis virus** (JEV), a mosquito-borne **flavivirus**, is a zoonotic pathogen that is prevalent in some Southeast Asian countries and causes acute encephalitis in humans. To evaluate the potential application of gene immunization to JEV infection, we characterized the immune responses from mice intramuscularly injected with plasmid DNA encoding JEV glycoproteins, including the precursor membrane (**prM**) plus envelope (**E**) proteins and the nonstructural protein NS1. When injected with the plasmid expressing **prM** plus **E**, 70% of the immunized mice survived after a lethal JEV challenge, whereas when immunized with the plasmid expressing NS1, 90% of the mice survived after a lethal challenge. As a control, the mice immunized with the DNA vector pcDNA3 showed a low level (40%) of protection, suggesting a nonspecific adjuvant effect of the plasmid DNA. Despite having no detectable neutralizing activity, the NS1 immunization elicited a strong antibody response exhibiting cytolytic activity against JEV-infected cells in a complement-dependent manner. By contrast, immunization with a construct expressing a longer NS1 protein (NS1'), containing an extra 60-amino-acid portion from the N terminus of NS2A, failed to protect mice against a lethal challenge. Biochemical analyses revealed that when individually expressed, NS1 but not NS1' could be readily secreted as a homodimer in large quantity and could also be efficiently expressed on the cell surface. Interestingly, when NS1 and NS1' coexisted in cells, the level of NS1 cell surface expression was much lower than that in cells expressing NS1 alone. These data imply that the presence of partial NS2A might have a negative influence on an NS1-based DNA vaccine. The results herein clearly illustrate that immunization with DNA expressing NS1 alone is sufficient to protect mice against a lethal JEV challenge.

L37 ANSWER 21 OF 83 MEDLINE on STN

1998037671. PubMed ID: 9371620. Naked DNA vaccines expressing the **prM** and **E** genes of Russian spring summer encephalitis virus and Central European encephalitis virus protect mice from homologous and heterologous challenge. Schmaljohn C; Vanderzanden L; Bray M; Custer D; Meyer B; Li D; Rossi C; Fuller D; Fuller J; Haynes J; Huggins J. (Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland 21702-5011, USA. ) Journal of virology, (1997 Dec) Vol. 71, No. 12, pp. 9563-9. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB Naked DNA vaccines expressing the **prM** and **E** genes of two tick-borne **flaviviruses**, Russian spring summer encephalitis (RSSE) virus and Central European encephalitis (CEE) virus were evaluated in mice. The vaccines were administered by particle bombardment of DNA-coated gold beads by Accell gene gun inoculation. Two immunizations of 0.5 to 1 microg of RSSE or CEE constructs/dose, delivered at 4-week intervals, elicited cross-reactive antibodies detectable by enzyme-linked immunosorbent assay and high-titer neutralizing antibodies to CEE virus. Cross-challenge experiments demonstrated that either vaccine induced protective immunity to homologous or heterologous RSSE or CEE virus challenge. The absence of antibody titer increases after challenge and the presence of antibodies to **E** and **prM**, but not NS1, both before and after challenge suggest that the vaccines prevented productive replication of the challenge virus. One vaccination with 0.5 microg of CEE virus DNA provided protective immunity for at least 2 months, and two vaccinations protected mice from challenge with CEE virus for at least 6 months.

L37 ANSWER 22 OF 83 MEDLINE on STN

1998006179. PubMed ID: 9347956. Rapid characterization of genetic diversity among twelve **dengue-2** virus isolates by single-strand conformation polymorphism analysis. Farfan J A; Olson K E; Black W C 4th; Gubler D J; Beaty B J. (Department of Microbiology, Colorado State University, Fort Collins 80523, USA. ) The American journal of tropical medicine and hygiene, (1997 Oct) Vol. 57, No. 4, pp. 416-22. Journal code: 0370507. ISSN: 0002-9637. Pub. country: United States. Language: English.

AB Single-strand conformation polymorphism (SSCP) analysis was used to characterize genetic polymorphisms among 12 isolates of **dengue-2** virus, which were previously genetically characterized by RNase T1 oligonucleotide mapping and by sequencing the viral envelope (**E**) gene.

reverse transcriptase-polymerase chain reaction. The viral **E**, premembrane (**prM**), and nonstructural 5 (NS5) gene cDNAs of 291 basepairs (bp), 291 bp, and 201 bp, respectively, were denatured, rapidly chilled to promote intrastrand reassociation, electrophoretically separated on nondenaturing polyacrylamide gels, and SSCP patterns were observed by silver staining. The SSCP analysis revealed polymorphisms among a number of **dengue**-2 isolates from the same topotype, and these were markedly different between isolates of different topotype (distinct genetic group). Comparison of nucleotide sequence and SSCP analyses of the 291-bp **E** cDNA demonstrated that virus isolates that produced identical SSCP patterns contained 0-7 nucleotide substitutions, whereas isolates that showed different SSCP patterns contained 4-25 nucleotide substitutions. Positive predictive value and negative predictive value as measures of certainty for predicting identical and different sequences were 26% and 100%, respectively. The SSCP patterns of the 12 **dengue**-2 isolates suggested greater genetic variation in the **prM** gene region than in either the **E** or NS5 gene regions. The SSCP analyses should allow easy, sensitive, and rapid screening of **dengue** viruses isolates and the assessment of variation at a number of sites in the virus genome. Additionally, SSCP screening of **dengue**-2 virus for genetic variability may reveal the introduction of new viral genotypes in a given geographic area. These genetic variants of the virus could serve as markers of the epidemic potential of the virus strain.

L37 ANSWER 23 OF 83 MEDLINE on STN

1998001369. PubMed ID: 9343204. Proteolytic activation of tick-borne encephalitis virus by furin. Stadler K; Allison S L; Schallich J; Heinz F X. (Institute of Virology, University of Vienna, Austria. ) Journal of virology, (1997 Nov) Vol. 71, No. 11, pp. 8475-81. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB **Flaviviruses** are assembled intracellularly in an immature form containing heterodimers of two envelope proteins, **E** and **prM**. Shortly before the virion exits the cell, **prM** is cleaved by a cellular enzyme, and this processing step can be blocked by treatment with agents that raise the pH of exocytic compartments. We carried out in vivo and in vitro studies with tick-borne encephalitis (TBE) virus to investigate the possible role of furin in this process as well as the functional consequences of **prM** cleavage. We found that **prM** in immature virions can be correctly cleaved in vitro by recombinant bovine furin but that efficient cleavage occurs only after exposure of the virion to mildly acidic pH. The data suggest that exposure to an acidic environment induces an irreversible structural change that renders the cleavage site accessible to the enzyme. Cleavage by furin in vitro resulted in biological activation, as shown by a 100-fold increase in specific infectivity, the acquisition of membrane fusion and hemagglutination activity, and the ability of the envelope proteins to undergo low-pH-induced structural rearrangements characteristic of mature virions. In vivo, **prM** cleavage was blocked by a furin inhibitor, and infection of the furin-deficient cell line LoVo yielded only immature virions, suggesting that furin is essential for cleavage activation of **flaviviruses**.

L37 ANSWER 24 OF 83 MEDLINE on STN

97335175. PubMed ID: 9191841. Changes in the **dengue** virus major envelope protein on passing and their localization on the three-dimensional structure of the protein. Lee E; Weir R C; Dalgarno L. (Division of Biochemistry and Molecular Biology, Faculty of Science, Australian National University, Canberra, Australia.. Eva.Lee@anu.edu.au) . Virology, (1997 Jun 9) Vol. 232, No. 2, pp. 281-90. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB To help define the molecular events involved in **dengue** virus adaptation during serial passage in vivo and in cultured cells, we have sequenced the structural protein genes of three **dengue** type 3 isolates after intracerebral passage in mice and after passage in cultured monkey kidney (Vero) and *Aedes albopictus* (mosquito) cells. Passaging in each host selected for amino acid changes in the envelope protein **E** and occasionally in **prM** but not in the capsid protein. Most changes were first apparent within five passages. Nineteen of twenty mutations in the structural protein genes resulted in amino acid changes concentrated on 12 residues; 9 of the 12 amino acid changes were at residues which are conserved between the four **dengue** virus serotypes. Certain amino acid changes were repeatedly selected on passage in cell culture. In six independent Vero cell passage series, changes were observed in **E** at residues 191 (four times), 202 (twice), 266 and 268 (three times), and 291; change in **prM** was seen in two passage series at residue 26. Two independent passage series in mosquito cells each resulted in the loss of a conserved glycosylation site at Asn 153 in **E**. Passage in mouse brain selected for mutations at **E** residues 18, 54, 277, 401, and 403. Residues which altered on passaging have been localized on the three-dimensional structure of the tick-borne encephalitis virus **E** protein soluble fragment (F. A. Rey, et al., 1995, Nature 375, 291-298). Residues 54, 191, 202, 266, 268, and 277 map to a postulated "hinge"

**flaviviruses** with cell membranes. The oligosaccharide at Asn 153 also appears to be involved in **flavivirus** fusion. Changes in the fusion characteristics of the passaged viruses were demonstrated.

=> d his

{FILE 'HOME' ENTERED AT 21:40:27 ON 01 JUN 2006}

FILE 'USPATFULL' ENTERED AT 21:40:50 ON 01 JUN 2006

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      E CHANG G J J/IN
      E CHANG GWONG J J/IN
L1      2 S E4
      E KONISHI E/AU
      E KONISHI E/IN
L2      24958 S CMV OR CMV-IE
L3      3918 S L2 AND KOZAK
L4      3136 S L3 AND TERMINATION
L5      0 S L4 AND (POLY W A)
L6      760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR
L7      33 S L6 AND CMV/CLM
L8      11 S L7 AND AY<1999
L9      498 S L6 AND PCDNA3?
L10     9 S L9 AND AY<1999
L11     3 S L10 NOT L8
      E SCHMALJOHN C S/IN
L12     12 S E4
L13     4840 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELLOW FEVER
L14     161 S L13 AND (PRM? AND E)
L15     82 S L14 AND (SIGNAL SEQUENCE)
L16     15 S L15 AND AY<1999
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FILE 'WPIDS' ENTERED AT 22:19:11 ON 01 JUN 2006

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      E CHANG G J J/IN
      E CHANG G J J/IN
L17     121 S E2
L18     3 S L17 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS O
L19     853 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITITS VIRU OR YELLOW
L20     28 S L19 AND (PRM? AND E)
L21     4 S L20 AND CMV
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FILE 'MEDLINE' ENTERED AT 22:28:38 ON 01 JUN 2006

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      E CHANG G J J/AU
L22     50 S E2
L23     23 S L22 AND (FLAVIVIR? OR DENGUE OR YELLOW FEVER OR JAPANESE ENCE
L24     16 S L23 AND PY<2000
      E KONISHI E/AU
L25     17 S E6
L26     6 S E5
      E KONISHI EIJI/AU
L27     25 S E2-E4
L28     16 S L27 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELL
L29     1144 S (JEV OR JAPANESE ENCEPHALITIS VIRUS)
L30     6 S L29 AND (SUBVIRAL PARTICLES)
L31     9634 S (FLAVIVIR? OR YELLOW FEVER VIRUS? OR JAPANESE ENCEPHALITIS VI
L32     1243 S L31 AND (PRM? OR E)
L33     233 S L32 AND (PRM?)
L34     194 S L33 AND (PRM AND E)
L35     88 S L34 AND PY<2000
L36     5 S L35 AND (SUBVIRAL PARTICLE? OR VIRUS-LIKE PARTICLE?)
L37     83 S L35 NOT L36
```

=> s l31 and (tetravalen? or multivalen?)

584 TETRAVALEN?

2863 MULTIVALEN?

L38 62 L31 AND (TETRAVALEN? OR MULTIVALEN?)

=> s l38 and py<2000

12412454 PY<2000

(PY<20000000)

L39 13 L38 AND PY<2000

=> d l39,cbib,ab,1-13

L39 ANSWER 1 OF 13 MEDLINE on STN

1999173489. PubMed ID: 10075167. The use of *Toxorhynchites splendens* for identification and quantitation of serotypes contained in the **tetravalent** live attenuated **dengue** vaccine. Jirakanjanakit N; Khin M M; Yoksan S; Bhamarapravati N. (Center for Vaccine Development, Mahidol University, Salaya, Nakornpathom, Thailand. ) Vaccine, (1999 Feb 12) Vol. 17, No. 6, pp. 597-601. Journal code: 8406899. ISSN: 0264-410X. Pub. country: ENGLAND: United Kingdom. Language: English.

control in the manufacture of vaccines. **Dengue-1** PDK13, **dengue-2** PDK53, **dengue-3** PGMK30F3 and **dengue-4** PDK48 in the live attenuated **tetravalent dengue** vaccine were assayed by identification and quantitation in a mosquito system (*Toxorhynchites splendens*). Each serotype of **dengue** virus was identified by **dengue** specific monoclonal antibodies in the indirect fluorescent antibody test. Virus content was estimated by calculating the 50% mosquito infectious dose (MID50). Differences from 0 to +/-0.5 log10 were observed between the original monovalent titer and that from the blend which showed no significant difference at 95% confidence limit ( $P < 0.05$ ). This result indicates that there is no interference between **dengue** serotypes in mosquitoes infected by intrathoracic inoculation with the virus mixture. It can be also concluded that this mosquito system can be used as an effective measure for infectivity titration of each component in the **tetravalent dengue** vaccine.

L39 ANSWER 2 OF 13 MEDLINE on STN

1998423756. PubMed ID: 9752834. **Dengue** and **dengue** haemorrhagic fever. Rigau-Perez J G; Clark G G; Gubler D J; Reiter P; Sanders E J; Vorndam A V. (Dengue Branch, Division of Vector-borne Infectious Diseases, Centers for Disease Control and Prevention, San Juan, Puerto Rico 00921-3200, USA.. Jorl@cdc.gov) . Lancet, (1998 Sep 19) Vol. 352, No. 9132, pp. 971-7. Ref: 83. Journal code: 2985213R. ISSN: 0140-6736. Pub. country: ENGLAND: United Kingdom. Language: English.

AB The incidence and geographical distribution of **dengue** have greatly increased in recent years. **Dengue** is an acute mosquito-transmitted viral disease characterised by fever, headache, muscle and joint pains, rash, nausea, and vomiting. Some infections result in **dengue** haemorrhagic fever (DHF), a syndrome that in its most severe form can threaten the patient's life, primarily through increased vascular permeability and shock. The case fatality rate in patients with **dengue** shock syndrome can be as high as 44%. For decades, two distinct hypotheses to explain the mechanism of DHF have been debated-secondary infection or viral virulence. However, a combination of both now seems to be the plausible explanation. The geographical expansion of DHF presents the need for well-documented clinical, epidemiological, and virological descriptions of the syndrome in the Americas. Biological and social research are essential to develop effective mosquito control, medications to reduce capillary leakage, and a safe **tetravalent** vaccine.

L39 ANSWER 3 OF 13 MEDLINE on STN

1998420053. PubMed ID: 9749624. Immunoglobulin M-capture biotin-streptavidin enzyme-linked immunosorbent assay for detection of antibodies to **dengue** viruses. Kittigul L; Suthachana S; Kittigul C; Pengruangrojanachai V. (Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok, Thailand. ) The American journal of tropical medicine and hygiene, (1998 Sep) Vol. 59, No. 3, pp. 352-6. Journal code: 0370507. ISSN: 0002-9637. Pub. country: United States. Language: English.

AB A biotin-streptavidin system was adapted to an IgM-capture ELISA for detection of **dengue** antibodies in human sera. To develop this assay, high titers of antibodies to **flavivirus** were purified by ion-exchange chromatography (DEAE-cellulose) and labeled with biotin. Heavy chain-specific goat anti-human IgM was first bound to the wells of a polystyrene microtiter plate, followed by binding of IgM in test specimens, and the use of **tetravalent dengue** antigens (**dengue** 1-4), biotin-labeled anti-**flavivirus** IgG, and streptavidin-peroxidase conjugate. The sensitivity and specificity of the IgM-capture biotin-streptavidin ELISA (IgM-BS-ELISA) in acute sera were 83.3% of patients with **dengue** infection and 95.3% of nondengue-infected cases, respectively. The positive predictive value was 92.4% and the negative predictive value was 89.2%. The efficiency of test was 90.4%. In convalescent sera, the sensitivity and specificity of IgM-BS-ELISA were 100% and 92.6%, respectively. The predictive values of positive and negative results were 90.3% and 100%, respectively. The efficiency of test was 95.6%. The agreement rate of IgM-BS-ELISA and standard hemagglutination inhibition test was good: kappa (kappa) values were 0.79 for acute sera and 0.91 for convalescent sera. The correlation between two methods was quite good, with correlation coefficients ( $r$ ) of 0.76 for acute sera and 0.85 for convalescent sera ( $P < 0.001$ ). The results indicate that the IgM-BS-ELISA is highly sensitive, specific, simple to perform, and rapid.

L39 ANSWER 4 OF 13 MEDLINE on STN

1998412674. PubMed ID: 9741643. Evaluation of molecular strategies to develop a live **dengue** vaccine. Lai C J; Bray M; Men R; Cahour A; Chen W; Kawano H; Tadano M; Hiramatsu K; Tokimatsu I; Pletnev A; Arakai S; Shameem G; Rinaldo M. (Molecular Viral Biology Section, Laboratory of Infectious Diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA.. clai@atlas.niaid.nih.gov) . Clinical and diagnostic virology, (1998 Jul 15) Vol. 10, No. 2-3, pp. 173-9. Journal code: 9309653. ISSN: 0928-0197. Pub. country: Netherlands.



AB BACKGROUND: Millions of individuals are estimated to become infected with **dengue** virus each year, particularly in tropical and subtropical regions. Mortality is low but infection can lead to a severe form of **dengue**, characterised by haemorrhage and shock. A safe and effective vaccine against **dengue** is still not available. OBJECTIVE: To use the successful construction of **dengue** type 4 virus (DEN4) cDNA, which yields infectious RNA transcripts, to provide a new approach to the development of safe and effective **dengue** vaccines. STUDY DESIGN: The 3' and 5' noncoding (NC) regions of the genome were targeted to construct DEN4 deletion mutants, because the sequences in these regions are thought to play an important role in the regulation of viral replication. DEN4 cDNA was also employed to construct a viable chimeric virus with **dengue** type 1, 2 or 3 antigenicity, by substitution of heterotypic structural protein genes. RESULTS: Most viable mutants, recovered from the cDNA constructs, were partially restricted for growth in simian cells as analysed by plaque morphology assay and viral yield analysis. Several 3' NC deletion mutants which exhibited a range of growth restriction in cell culture were further evaluated for infectivity and immunogenicity in rhesus monkeys. Occurrence and duration of viraemia were reduced for these deletion mutants, compared to the wild type DEN4. Analysis of antibody response to infection in rhesus monkeys also indicated that some of these mutants were attenuated. These DEN4 deletion mutants represent promising live **dengue** vaccine candidates that merit further clinical evaluation. Chimera DEN1/DEN4 or DEN2/DEN4 which expresses DEN1 or DEN2 antigenicity were also used to infect monkeys. Most monkeys immunised with these chimeric viruses, singly or in combination, developed high titres of neutralising antibodies and were protected against homotypic wild type DEN1 or DEN2 challenge. CONCLUSIONS: DEN4 and its derived chimeric viruses of other three **dengue** serotype specificity, that contain appropriate attenuating mutations, have a potential use in a **tetravalent** live vaccine against **dengue**.

L39 ANSWER 5 OF 13 MEDLINE on STN  
 1998033180. PubMed ID: 9367357. Analysis of a recombinant **dengue**-2 virus-**dengue**-3 virus hybrid envelope protein expressed in a secretory baculovirus system. Bielefeldt-Ohmann H; Beasley D W; Fitzpatrick D R; Aaskov J G. (Centre for Molecular Biotechnology, School of Life Science, Queensland University of Technology, Brisbane, Australia.. helle@biosci.uq.edu.au) . The Journal of general virology, (1997 Nov) Vol. 78 ( Pt 11), pp. 2723-33. Journal code: 0077340. ISSN: 0022-1317. Pub. country: ENGLAND: United Kingdom. Language: English.

AB In a step towards a **tetravalent dengue** virus subunit vaccine which is economical to produce, highly immunogenic and stable, a hybrid **dengue** virus envelope (E) protein molecule has been constructed. It consists of 36 amino acids from the membrane protein, the N-terminal 288 amino acids of the **dengue**-2 virus E protein plus amino acids 289-424 of the **dengue**-3 virus E protein. It has been engineered for secretory expression by fusion to a mellitin secretory signal sequence and truncation of the hydrophobic transmembrane segment. Using the baculovirus expression system and serum-free conditions, more than 95% of recombinant **dengue**-2 virus-**dengue**-3 virus hybrid E protein (rD2D3E) was secreted into the cell culture supernatant in a stable form with multiple features indicative of preserved conformation. The hybrid molecule reacted with a panel of **dengue** virus- and **flavivirus**-specific MAbs which recognize linear or conformational epitopes on **dengue** virions. Human **dengue** virus-specific antisera also reacted with the protein. The hybrid rD2D3E protein was able to inhibit the in vitro binding of **dengue**-2 and **dengue**-3 viruses to human myelomonocytic cells, suggesting that the receptor-binding epitope(s) was preserved. Adjuvant-free immunization with the hybrid protein induced an antibody response to both **dengue**-2 and **dengue**-3 virus in outbred mice, comparable in strength to that of individual rD2E and rD3E proteins. Notably, these antibody responses were primarily of the IgG2a and IgG2b isotype. A strong **dengue** virus cross-reactive T cell response was also induced in the mice, suggesting that **dengue** virus hybrid E proteins could form the basis of an efficacious **multivalent dengue** virus vaccine.

L39 ANSWER 6 OF 13 MEDLINE on STN  
 96215657. PubMed ID: 8645110. Immunisation with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. Phillpotts R J; Venugopal K; Brooks T. (Microbiology Group, Chemical and Biological Defence Establishment, Porton Down, Wiltshire, U.K. ) Archives of virology, (1996) Vol. 141, No. 3-4, pp. 743-9. Journal code: 7506870. ISSN: 0304-8608. Pub. country: Austria. Language: English.

AB In vivo transfection by intramuscular injection with plasmids expressing the immunogenic proteins of microbial pathogens has considerable potential as a vaccination strategy against many pathogens of both man and animals. Here we report that weanling mice given a single intramuscular injection of 50 micrograms of a plasmid, pSLE1 expressing the St. Louis encephalitis virus (SLE) prM/E protein under the control of the cytomegalovirus immediate early protein promoter produced SLE-specific antibody and were protected against lethal challenge with the virulent

produce vaccines against **flavivirus** diseases of low incidence cheaply and rapidly, and to produce **multivalent** vaccines such as would be required for immunisation against **dengue** virus disease.

L39 ANSWER 7 OF 13 MEDLINE on STN

96211559. PubMed ID: 8648761. Monkeys immunized with intertypic chimeric **dengue** viruses are protected against wild-type virus challenge. Bray M; Men R; Lai C J. (Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892, USA. ) Journal of virology, (1996 Jun) Vol. 70, No. 6, pp. 4162-6. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB **Dengue** epidemics caused by the four **dengue** virus serotypes continue to pose a major public health problem in most tropical and subtropical regions. A safe and effective vaccine against **dengue** is still not available. The current strategy for **dengue** immunization favors the use of a vaccine containing each of the four serotypes. We previously employed full-length **dengue** type 4 virus (DEN4) cDNA to construct a viable intertypic **dengue** virus of type 1 or type 2 antigenic specificity that contained the genes for the capsid-premembrane-envelope (C-pre-M-E) structural proteins of DEN1 or pre-M and E structural proteins of DEN2 substituting for the corresponding DEN4 genes. Chimeras DEN1/DEN4 and DEN2/DEN4, which express the nonstructural proteins of DEN4 and the C-pre-M-E structural proteins of DEN1 or the pre-M-E structural proteins of DEN2, and therefore the antigenicity of type 1 or type 2, were used to immunize rhesus monkeys. Other monkeys were inoculated with parental DEN1, DEN2, or cDNA-derived DEN4. Three of four monkeys immunized with DEN1/DEN4 developed neutralizing antibodies against DEN1 and were protected against subsequent DEN1 challenge. All four monkeys immunized with DEN2/DEN4 developed antibodies against DEN2 and were protected against subsequent DEN2 challenge. DEN1- and DEN2-immunized monkeys were protected against homologous virus challenge, but DEN4-immunized animals became viremic on cross-challenge with DEN1 or DEN2. In a second experiment, eight monkeys were immunized with equal mixtures of DEN1/DEN4 and DEN2/DEN4. Each of these monkeys developed neutralizing antibodies against both DEN1 and DEN2 and were protected against subsequent challenge with DEN1 or DEN2. Chimeric **dengue** viruses similar to those described here could be used to express serotype-specific antigens in a live attenuated **tetravalent** human vaccine.

L39 ANSWER 8 OF 13 MEDLINE on STN

96048320. PubMed ID: 8551257. Rapid and sensitive streptavidin-biotin amplified fluorogenic enzyme-linked immunosorbent-assay for direct detection and identification of **dengue** viral antigens in serum. Malergue F; Chungue E. (Unite de Virologie, Institut Territorial de Recherches Medicales Louis Malarde, Tahiti, French Polynesia. ) Journal of medical virology, (1995 Sep) Vol. 47, No. 1, pp. 43-7. Journal code: 7705876. ISSN: 0146-6615. Pub. country: United States. Language: English.

AB Each of the four serotypes of **dengue** viruses is responsible for a spectrum of illnesses that range from nonspecific febrile syndrome with good prognosis to **dengue** haemorrhagic fever or **dengue** shock syndrome. Definite diagnosis of **dengue** is provided by the detection of virus in acute-phase sera of patients. Virus isolation can be accomplished with mosquito cell lines or mosquito inoculations. However, these methods are time consuming and labour intensive. The reverse-transcriptase polymerase chain reaction (RT-PCR) provides a potential means of rapid diagnosis but requires specialised facilities and equipment and is expensive. Therefore a rapid, simple, sensitive, and economical method for direct detection of viral antigens in viraemic sera is needed for clinical and epidemiological investigations. An amplified fluorogenic enzyme-linked immunosorbent assay (F-ELISA) is described for the detection and identification of **dengue**-3 viruses in serum specimens. This assay utilizes biotinylated mouse IgG antibody directed against **dengue** antigens captured by anti-**dengue** monoclonal antibody coated onto polystyrene microplate wells. It takes advantage of the high affinity of biotin for the **multivalent** binding sites of streptavidin-labelled beta-galactosidase, and combines the amplification effect of biotin-streptavidin interaction with the high sensitivity of fluorogenic detection methods. Following optimisation of the procedure by reducing non-specific binding of proteins and enhancing the specific binding of antigens, F-ELISA was tested on 259 sera submitted routinely to our laboratory for confirmation of **dengue** diagnosis. The sensitivity of the F-ELISA was 90%, the specificity was 99% and the agreement rate was 98% between F-ELISA and virus isolation results.

L39 ANSWER 9 OF 13 MEDLINE on STN

95333308. PubMed ID: 7609092. Construction of intertypic chimeric **dengue** viruses exhibiting type 3 antigenicity and neurovirulence for mice. Chen W; Kawano H; Men R; Clark D; Lai C J. (Molecular Viral Biology Section, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892, USA. ) Journal of virology, (1995 Aug) Vol. 69, No. 8, pp. 5186-90. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

major epidemics in tropical or subtropical areas. The current strategy for **dengue** virus immunization favors the use of a **tetravalent** vaccine preparation. We have previously employed full-length DEN4 cDNA to construct a viable intertypic **dengue** virus type 1 or type 2 chimera that contained the C-PreM-E or only the PreM-E genes of DEN1 or DEN2 substituting for the corresponding genes of DEN4. This success implied that it might be possible to create mutants of all four **dengue** virus serotypes for evaluation as candidate vaccines. In this study, we constructed DEN3-DEN4 chimeras that contained DEN3 C-PreM-E genes and expressed DEN3 antigenic specificity. Unlike our previous successes in cloning DEN1 or DEN2 chimeric cDNA, we were not able to clone the DEN3 C-PreM-E genes directly in the 5' intermediate vector or in the full-length chimeric DEN3-DEN4 plasmid in *Escherichia coli*. Nevertheless, a full-length DNA template of DEN3-DEN4 that could be used for transcription of infectious RNAs was prepared by in vitro ligation. Progeny virus recovered from RNA-transfected C6/36 mosquito cells exhibited DEN3 antigenic specificity as determined by a reaction with monoclonal antibodies. Gel electrophoresis of virus-infected cell lysates yielded the predicted viral protein pattern, i.e., DEN3 C, PreM, and E and DEN4 nonstructural proteins. Two amino acid substitutions, Thr-435-->Leu and Glu-406-->Lys, which are analogous to mutations that, respectively, confer mouse neurovirulence on DEN4 and DEN2, were introduced into DEN3 E. A mutant chimera containing the Thr-435-->Leu substitution, which ablates the potential glycosylation site sequence, produced an E protein identical in size to that of wild-type DEN3 E, indicating that the glycosylation site is normally not used. Intracerebral inoculation of suckling mice revealed that the mutant chimera containing the Glu-406-->Lys substitution was neurovirulent, whereas its wild-type counterpart or parent DEN3 was not.

L39 ANSWER 10 OF 13 MEDLINE on STN

94339727. PubMed ID: 8061530. [A serological survey regarding **Flaviviridae** infections on the island of Reunion (1971-1989)]. Enquetes serologiques concernant les arboviroses a **Flaviviridae** sur l'île de la Reunion (1971-1989). Kles V; Michault A; Rodhain F; Mevel F; Chastel C. (Faculte de Medecine de Brest, Departement de Microbiologie et de Sante Publique, France. ) Bulletin de la Societe de pathologie exotique (1990), (1994) Vol. 87, No. 2, pp. 71-6. Journal code: 9212564. ISSN: 0037-9085. Pub. country: France. Language: French.

AB Serological prevalence of **Flaviviridae** was studied on Reunion Island by testing 2,507 human sera from a randomised sample. Each serum was tested against 5 viruses (yellow fever, **dengue** type 1 and 2, West Nile and Wesselsbron) using haemagglutination inhibition test: 42.68% of human sera were found positive. The **multivalent** reactions represent practically three fourths of the positive ones. A severe **dengue** type 2 outbreak on the island in 1977-1978 and the possible circulation of a **Flavivirus** may explain these data. Among the tested subjects, positivity frequencies vary according to their age and their living surrounding. These results were compared with those of a previous serosurvey performed in 1971 and showed an important increase in the serological prevalence largely explainable by the 1977-1978 epidemic.

L39 ANSWER 11 OF 13 MEDLINE on STN

94242192. PubMed ID: 8185773. Children's vaccine initiative. Anonymous. World health forum, (1993) Vol. 14, No. 2, pp. 202-3. Journal code: 8010746. ISSN: 0251-2432. Report No.: PIP-083243; POP-00223785. Pub. country: Switzerland. Language: English.

AB The Consultative Group of the Children's Vaccine Initiative (CVI) held its annual meeting at WHO headquarters in Geneva in November 1992. The CVI meeting provided evidence of solid progress in development of a thermostable oral poliomyelitis vaccine and a single-dose tetanus toxoid vaccine. Researchers were able to stabilize the vaccine for several days at 37 degrees Celsius; this could make a more thermostable oral poliomyelitis vaccine available to the public before the end of the decade. One research project had solved the problem of stability of microencapsulated tetanus toxoid at 37 degrees Celsius. Based on recent research, it appears that microspheres for delivery of tetanus toxoid vaccines (of different sizes and/or compositions) can induce long-lasting immunity. The Consultative Group hopes that experts will conduct the industrial development of these vaccines within 3 years. A comprehensive database of the world's capacity to produce children's vaccines is being compiled, and investment strategies will assure that affordable vaccines are available for all children of the world. For the past 13 years, WHO has supported research at the Mahidol University in Bangkok, Thailand, to develop a safe, immunogenic, live, attenuated, **tetravalent** vaccine against the 4 strains of **dengue** virus. Formal Phase 1 and Phase 2 clinical trials have proved the vaccine to be safe and immunogenic in humans, thus bringing the **Dengue** Vaccine Development Project to a successful conclusion. Researchers are preparing to test the vaccine under actual field conditions for its efficacy in preventing **dengue** and **dengue** hemorrhagic fever among children in endemic communities.

the Netherlands, in October 1992 adopted a World Declaration on the control of malaria which will serve as a blueprint for action in the 1990s for a partnership of malaria-endemic and malaria-free countries.

L39 ANSWER 12 OF 13 MEDLINE on STN

93381798. PubMed ID: 8371350. **Dengue** virus-specific human CD4+ T-lymphocyte responses in a recipient of an experimental live-attenuated **dengue** virus type 1 vaccine: bulk culture proliferation, clonal analysis, and precursor frequency determination. Green S; Kurane I; Edelman R; Tacket C O; Eckels K H; Vaughn D W; Hoke C H Jr; Ennis F A. (Department of Medicine, University of Massachusetts Medical Center, Worcester 01655. ) Journal of virology, (1993 Oct) Vol. 67, No. 10, pp. 5962-7. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB We analyzed the CD4+ T-lymphocyte responses to **dengue**, West Nile, and **yellow fever viruses** 4 months after immunization of a volunteer with an experimental live-attenuated **dengue** virus type 1 vaccine (DEN-1 45AZ5). We examined bulk culture proliferation to noninfectious antigens, determined the precursor frequency of specific CD4+ T cells by limiting dilution, and established and analyzed CD4+ T-cell clones. Bulk culture proliferation was predominantly **dengue** virus type 1 specific with a lesser degree of cross-reactive responses to other **dengue** virus serotypes, West Nile virus, and **yellow fever virus**. Precursor frequency determination by limiting dilution in the presence of noninfectious **dengue** virus antigens revealed a frequency of antigen-reactive cells of 1 in 1,686 peripheral blood mononuclear cells (PBMC) for **dengue** virus type 1, 1 in 9,870 PBMC for **dengue** virus type 3, 1 in 14,053 PBMC for **dengue** virus type 2, and 1 in 17,690 PBMC for **dengue** virus type 4. Seventeen CD4+ T-cell clones were then established by using infectious **dengue** virus type 1 as antigen. Two patterns of **dengue** virus specificity were found in these clones. Thirteen clones were **dengue** virus type 1 specific, and four clones recognized both **dengue** virus types 1 and 3. Analysis of human leukocyte antigen (HLA) restriction revealed that five clones are HLA-DRw52 restricted, one clone is HLA-DP3 restricted, and one clone is HLA-DP4 restricted. These results indicate that in this individual, the CD4+ T-lymphocyte responses to immunization with live-attenuated **dengue** virus type 1 vaccine are predominantly serotype specific and suggest that a **multivalent** vaccine may be necessary to elicit strong serotype-cross-reactive CD4+ T-lymphocyte responses in such individuals.

L39 ANSWER 13 OF 13 MEDLINE on STN

91171791. PubMed ID: 2077321. [40 cases of **dengue** (serotype 3) occurring in a military camp during an epidemic in New Caledonia (1989). The value of vector control]. A propos de 40 cas de **dengue** (serotype 3) survenus dans un camp militaire lors de l'epidemie de Nouvelle-Caledonie (1989). Interet de la lutte antivectorielle. Berard H; Laille M. (Medecin des Armees, Noumea (Nouvelle-Caledonie). ) Medecine tropicale : revue du Corps de sante colonial, (1990 Oct-Dec) Vol. 50, No. 4, pp. 423-8. Journal code: 8710146. ISSN: 0025-682X. Pub. country: France. Language: French.

AB An epidemic of **dengue** occurred at the beginning of 1989 in New Caledonia. About 18 p.c. of the population was stricken (25,000 to 30,000 estimated clinical cases). The military camp of Plum was stricken too, but a prompt vector control was established. 8.6 p.c. of the strength was affected by classical **dengue** without severe hemorrhagic manifestations. Such prevalence is lower than the one rated in the civil population, demonstrating the major importance of vector control to limit spreading of such an epidemic as a **tetravalent** vaccine is not yet available.

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E CHANG GWONG J J/IN
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L2      24958 S CMV OR CMV-IE
L3      3918 S L2 AND KOZAK
L4      3136 S L3 AND TERMINATION
L5      0 S L4 AND (POLY W A)
L6      760 S L4 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS VIRUS OR
L7      33 S L6 AND CMV/CLM
L8      11 S L7 AND AY<1999
L9      498 S L6 AND PCDNA3?
L10     9 S L9 AND AY<1999
L11     3 S L10 NOT L8
        E SCHMALJOHN C S/IN
L12     12 S E4

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L14 161 S L13 AND (PRM? AND E)  
L15 82 S L14 AND (SIGNAL SEQUENCE)  
L16 15 S L15 AND AY<1999

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L17 121 S E2  
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L19 853 S (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITITS VIRU OR YELLOW  
L20 28 S L19 AND (PRM? AND E)  
L21 4 S L20 AND CMV

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L23 23 S L22 AND (FLAVIVIR? OR DENGUE OR YELLOW FEVER OR JAPANESE ENCE  
L24 16 S L23 AND PY<2000  
E KONISHI E/AU  
L25 17 S E6  
L26 6 S E5  
E KONISHI EIJI/AU  
L27 25 S E2-E4  
L28 16 S L27 AND (FLAVIVIR? OR DENGUE OR JAPANESE ENCEPHALITIS OR YELL  
L29 1144 S (JEV OR JAPANESE ENCEPHALITIS VIRUS)  
L30 6 S L29 AND (SUBVIRAL PARTICLES)  
L31 9634 S (FLAVIVIR? OR YELLOW FEVER VIRUS? OR JAPANESE ENCEPHALITIS VI  
L32 1243 S L31 AND (PRM? OR E)  
L33 233 S L32 AND (PRM?)  
L34 194 S L33 AND (PRM AND E)  
L35 88 S L34 AND PY<2000  
L36 5 S L35 AND (SUBVIRAL PARTICLE? OR VIRUS-LIKE PARTICLE?)  
L37 83 S L35 NOT L36  
L38 62 S L31 AND (TETRAVALEN? OR MULTIVALEN?)  
L39 13 S L38 AND PY<2000

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PATENT

Attorney Reference Number 6395-64907-01  
Application Number 09/701,536

WO 93/06214

188(2):  
180:401 1991  
188:714 1992

LRW SPRC.

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5494671

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1-34 (canceled)

188:72(5):4925 1998

Claims

35-54, 69

E. KOWISHI 1991/1992

35. (previously presented) An isolated nucleic acid comprising a transcriptional unit for an immunogenic flavivirus antigen, wherein the transcriptional unit directs a host cell, after being incorporated therein, to synthesize the immunogenic antigen, and wherein the transcriptional unit comprises a prM signal sequence and a Kozak ribosomal binding sequence located in a position that is effective for ribosome binding.

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36. (previously presented) The nucleic acid of claim 35, wherein the flavivirus comprises yellow fever virus, dengue serotype 1 virus, dengue serotype 2 virus, dengue serotype 3 virus, dengue serotype 4 virus, St. Louis encephalitis virus, Japanese encephalitis virus, or a mixture of two or more thereof.

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37. (previously presented) The nucleic acid of claim 35, wherein the antigen is a prM/M protein, an E protein, or both a prM/M protein and an E protein.

MULTIPLAUNT

38. (previously presented) The nucleic acid of claim 37, wherein the antigen is both the prM/M protein and the E protein and wherein the host cell secretes subviral particles comprising the prM/M protein and the E protein.

TETRAVAUNT

39. (previously presented) The nucleic acid of claim 35 which is DNA.

prM + E

SIGNAL SEQUENCE

40. (previously presented) The nucleic acid of claim 35, wherein the transcriptional unit further comprises a control sequence disposed appropriately such that it operably controls synthesis of the antigen.

VIRUS-LIKE PARTICLES

SUBVIRAL PARTICLES

41. (previously presented) The nucleic acid of claim 40, wherein the control sequence is the cytomegalovirus immediate early promoter.

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Page 2 of 7



prM/M

REV  
SIGNAL  
PART

42. (previously presented) The nucleic acid of claim 35, wherein the transcriptional unit further comprises a poly-A terminator.

43. (previously presented) A cell comprising the nucleic acid of claim 35.

44. (previously presented) The cell of claim 43, wherein the flavivirus comprises yellow fever virus, dengue serotype 1 virus, dengue serotype 2 virus, dengue serotype 3 virus, dengue serotype 4 virus, St. Louis encephalitis virus, Japanese encephalitis virus, or a mixture of two or more thereof.

45. (previously presented) The cell of claim 43, wherein the flavivirus antigen is a prM/M protein, an E protein, or both a prM/M protein and an E protein.

46. (previously presented) The cell of claim 45, wherein the antigen is both the prM/M protein and the E protein and wherein the cell secretes subviral particles comprising the prM/M protein and E protein.

47. (previously presented) A composition comprising the nucleic acid of claim 35 in a pharmaceutically acceptable carrier.

48. (previously presented) The composition of claim 47, wherein the flavivirus comprises yellow fever virus, dengue serotype 1 virus, dengue serotype 2 virus, dengue serotype 3 virus, dengue serotype 4 virus, St. Louis encephalitis virus, Japanese encephalitis virus, or a mixture of two or more thereof.

49. (previously presented) The composition of claim 47, wherein the antigen is a prM/M protein, an E protein, or both a prM/M protein and an E protein.

50. (previously presented) The composition of claim 49, wherein the antigen is both the prM/M protein and the E protein and wherein the cell secretes subviral particles comprising the prM/M

protein and the E protein.

51. (previously presented) The composition of claim 47, wherein the nucleic acid is DNA.

52. (previously presented) The composition of claim 47, wherein the transcriptional unit further comprises a control sequence disposed appropriately such that it operably controls synthesis of the antigen.

53. (previously presented) The composition of claim 52, wherein the control sequence is the cytomegalovirus immediate early promoter.

54. (previously presented) The composition of claim 47, wherein the transcriptional unit further comprises a poly-A terminator.

55 – 68 (canceled)

69. (previously presented) The nucleic acid of claim 35, wherein the Kozak ribosomal binding sequence is located from positions -9 to +4 in the transcriptional unit.

70 – 86 (canceled)



# ANALYSIS OF JAPANESE ENCEPHALITIS (JE) VIRUS GENOME AND IMPLICATIONS FOR RECOMBINANT JE VACCINE

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<sup>1</sup>Department of Microbiology, Tokyo Metropolitan Institute for Neurosciences, Musashidai, Fuchu, Tokyo, <sup>2</sup>Biological Science Laboratory, Nippon Zeon, Kawasaki, <sup>3</sup>National Institute of Health, Tokyo, <sup>4</sup>Chiba Serum Institute, Chiba, Japan.

**Abstract.** From the information of nucleotide sequences and deduced amino acid sequences of flaviviruses including JEV, we can postulate processing mechanisms of a polyprotein translated from single long open reading frame of the genome and mechanisms of construction of antigenic structures of structural proteins with biologically active forms after these proteins are translated.

The results of comparative analysis of amino acid sequences among flaviviruses and epitope analysis on the E proteins which are the most important antigens for protective immunity suggest that the E protein of flaviviruses may have a similar structure closely related to each other.

PrM and E proteins which had predictable signal sequences upstream on the N terminals were expressed with antigenically active form and molecular size the same as the authentic ones by the recombinant viruses. However, the recombinant viruses which had no such signal sequence expressed unprocessed proteins with antigenically denatured forms. These results suggest that normal proteolytic processing is needed to construct biologically active structures of JEV structural proteins.

The E proteins which were expressed by the recombinant viruses as antigenically active form could elicit neutralizing and HI antibodies in animals and protective immunity in mice. The recombinant vaccinia viruses which express the E protein could induce strong immunologic memory against the E protein in mice. These results indicate that the development of a new type of vaccine against JEV will become possible in future.

## INTRODUCTION

Japanese encephalitis virus (JEV) is the most important mosquito-borne human pathogen causing encephalitis in southeastern and far eastern Asia. It has recently extended its range westward into India. JEV belongs to the family *Flaviviridae* which contains more than 60 antigenically related viruses having a single molecule of plus-sense RNA genome (Westaway *et al*, 1985). The flavivirus RNA consists of about 11,000 nucleotides which encodes a single long open reading frame (Rice *et al*, 1985). It is thought to be translated to a long polyprotein which is subsequently cleaved into three struc-

tural proteins, designated C, M and E proteins, and several nonstructural proteins.

An effective inactivated JEV vaccine has been used for over 2 decades in Japan, Taiwan, and Korea, and JE has been considerably well controlled in these countries (Oya, 1988). In the endemic areas, however, JE is still a serious problem for humans and cattle; these areas have other flavivirus diseases too, and a safe, effective vaccine is needed. The possibility that the immunity against one kind of flavivirus produces crucial effects on another flavivirus infection has been considered. The development of a new vaccine may be made possible by understanding of the mechanism of protection

Vol 21 No 4 December 1990

663

SIGNAL SEQUENCES

JEV PrM/E

PrM SEG. SEQ. REQUIRED FOR OPTIMAL PROCESSING

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against the flaviviruses. In flaviviruses, the E protein appears to play an important role in inducing protective immunity against flavivirus infection in animals as well as humans (Russell *et al*, 1980.) Previous studies have suggested that the E protein of JEV possesses three different kinds of antigenic determinants based on its cross-reactivity with flaviviruses; they are cross-reactive, subgroup specific, and serotype specific. Monoclonal antibodies to the E glycoprotein have been characterized (Kimura-Kuroda and Yasui, 1983) and divided into nine groups (Kimura-Kuroda and Yasui, 1986; Kimura-Kuroda and Yasui, 1988) based on hemagglutination inhibition (HI), neutralization, and reactivity to virions. These findings suggest that expression of the E protein of JEV in a biologically active form may be most conducive to the development of a new JE vaccine.

### JEV GENOME STRUCTURE

The nucleotide sequences of many members of flaviviridae have been reported since that of the yellow fever virus was determined firstly by Rice *et al* (1985). The first report of complete nucleotide sequence of JEV was done by Sumiyoshi *et al* (1987). JEV genome of a typical strain is composed of 10976 nucleotides which encodes a single long open reading frame of 10296 nucleotides corresponding 3432 amino acid residues. This long polypeptide is thought to be cleaved co-translationally into three structural proteins and 7 non-structural proteins. The junctions between C and prM, M and E, E and NS1, and ns4a and ns4b are supposed to be cleaved by cellular signalase. The junction between NS1 and ns2a may be cleaved by ns2a in a cis manner. The possibility is postulated that the junctions between ns2a and ns2b, ns2b and NS3, NS3 and ns4a, and ns4b and NS5 are cleaved by NS3 in a cis manner. Mature virion has C, M, and E proteins. So, the prM protein may be cleaved to M during maturational morphogenesis of the virion (Fig 1). The nucleotide and deduced amino acid sequences of several strains of

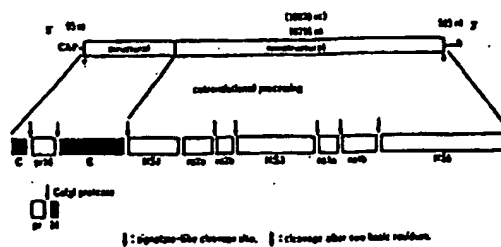


Fig 1—JE virus genome and proteins.

JEV have been compared each other and are highly conserved.

The nucleotide sequences of *Pestivirus* have been recently reported (Collet *et al*, 1988), and now we also know the nucleotide sequence of the entire coding region of nonA, nonB hepatitis virus, that is HCV. These two viruses have a plus sense RNA for the genomes and a single long open reading frame the same as that of flaviviruses. Hydrophobicity profiles of structural and non-structural proteins of these three viruses resemble each other. Homology of amino acid sequences among these viruses are scarcely observed, however, we can find 7 homology boxes of amino acid sequence in NS3 region and 2 homology boxes in NS5 region among these 3 viruses. So, we will have to classify these 3 viruses into the same family.

### JEV E PROTEIN STRUCTURE

The E protein of flaviviruses plays an important role in inducing protective immunity against flavivirus infection. The development of a new vaccine may be made possible by understanding of the E protein structure.

Nine groups of epitopes on the E protein of JEV have been defined with monoclonal antibodies and characterized based on HI, neutralization, reactivity to virions, and competitive binding with each other to virions (Kimura-Kuroda and Yasui, 1983; Kimura-Kuroda and Yasui, 1986; Kimura-Kuroda and Yasui, 1988). Antibodies against these epitopes show different cross-reactivities with flavi-

viruses: they are flavivirus cross-reactive, subgroup virus specific, serotype specific, and strain specific.

Basic structures of the E protein of flaviviruses may resemble each other, because there are 3 highly conserved areas of amino acid sequence and almost all cysteine residues and many proline residues are well conserved among flaviviruses. Homologies of amino acid sequence among the JEV subgroup viruses causing encephalitis are very high. Homology between JEV and Murray valley encephalitis virus is more than 80% and homology between JEV and St Louis encephalitis virus, which shows lowest homology in the JEV subgroup is nevertheless about 71%. However, homologies between JEV and dengue viruses are about 45%. Detailed structures of dengue virus E proteins may differ from that of JEV.

These data were confirmed by the results of homology relationships of the epitopes among flaviviruses based on cross-reactivities of mo-

noclonal antibodies. All subgroup reactive monoclonal antibodies against same group epitope cross-reacted with Murray valley encephalitis virus, however, few antibodies among them reacted with St Louis encephalitis virus. Table 1 is a summary of epitopes on the E protein of JEV. Those epitope groups shown in Table 1 are arranged according to topographical relationships with each other from the results of competitive binding analysis of the monoclonal antibodies. The area constructed with group 8 epitopes showed high neutralizing activity and neighboring epitopes on the E protein have virus species specific characters.

Characteristic hydrophobic domains are observed at the C terminal ends of the E and M proteins by hydrophobicity analysis of the amino acid sequences of these proteins. The E and M proteins may be anchored to the envelope of the virion with these C terminal hydrophobic domains. Many cysteine residues cluster at the N terminal half of the E protein and many

Table 1

Summary of epitopes on the E protein of JEV.

Epitopes recognized by	Biological functions against JEV*			Cross-reactivity**
MAB group 1	HI ++	N -	PA +	Flavivirus cross-reactive
2	HI ++	N -	PA +	Subgroup reactive
3	HI -	N +	PA ++	JEV specific, strain specific
8	HI -	N +++	PA +++	JEV specific
7	HI ++	N ++	PA ++	Subgroup reactive
6	HI -	N -	PA -	Subgroup reactive
5	HI -	N +	PA +	Subgroup reactive
4	HI -	N +	PA -	Subgroup reactive
9	HI -	N -	PA -	Flavivirus cross-reactive
(S1)	HI -	N -	NT	Strain specific
(S2)	NT	N +	NT	Strain specific

\* HI = hemagglutination inhibition, N = neutralizing activity, PA = protective activity against JEV infection on mice, NT = not tested.

\*\* Cross-reactivities were determined against yellow fever, dengue (types 1, 2, 3 and 4) and JEV subgroup (Murray valley encephalitis, West Nile and St Louis encephalitis) viruses.

proline residues cluster at the C terminal half. These findings, including those of the epitope analysis, suggest that expression of the E protein of JEV in a biologically active form may be most conducive to the development of a new JE vaccine.

### EXPRESSION OF THE STRUCTURAL PROTEIN E

In order to investigate the relationship between processing and construction of antigenic structure on the structural proteins, we made recombinant baculo and vaccinia viruses containing the coding sequences of structural proteins and analysed the expressed proteins. The prM, E, and NS1 proteins have signal sequences upstream from the coding sequence of each protein, respectively. We made several different constructs which had or did not have a signal sequence upstream from the coding sequence of each protein or had truncated structural protein genes. The polyhedrin promoter of *Autographa californica* polyhedrosis virus was used and recombinant baculoviruses were selected from the virus plaques after DNAs of the virus and transfer vector containing the JEV sequence were co-transfected into *Spodoptera frugiperda* cells (Matsuura *et al*, 1989). To make recombinant vaccinia viruses, the coding sequences of JEV structural proteins were inserted downstream at the 7.5K promoter of the transfer vector and recombined at the TK region of vaccinia virus (Yasuda *et al*, 1990).

The proteins expressed by the recombinant viruses were analysed by immunoprecipitation, Western blotting, immunofluorescent antibody, fluorescence-activated flow cytometry and ELISA methods. Antigenic properties of the proteins expressed by the recombinant viruses were evaluated using a panel of monoclonal antibodies and several kinds of antisera. Similar results were obtained for recombinant baculoviruses and vaccinia viruses. The JEV proteins expressed by the recombinants could be detected in all cases with mouse antisera against JEV-infected mouse brain homogenate dena-

tured with SDS or antibody against SDS-denatured E protein. However, with antibody against native JEV or monoclonal antibodies against native E protein, only the antigens expressed by the recombinants which had the signal sequence upstream were detected. And these antigens were processed considerably. The E proteins were also detected on the cell surfaces of the well processed cases which were infected by the recombinants with the signal sequence (Fig 2). These results show that processing of the expressed proteins, construction of the native antigenic structures and expression of the antigens on the infected cell surface proceed in parallel. Concerning epitope expression of the E, both protective epitopes, groups 8 and 7, were considerably expressed by the re-

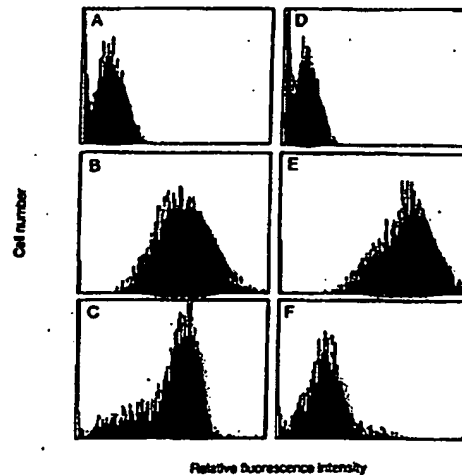


Fig 2—Fluorescence-activated flow cytometric analysis of JEV antigens on Vero cell surfaces infected with vaccinia virus LC16mO (A and D), JEV (B and E), or recombinant vaccinia virus J6 (C and F). Virus-infected cells were reacted with anti-JEV serum (A to C) or monoclonal antibody N.04 (D to F), incubated with a fluorescein conjugated goat anti-rabbit or anti-mouse secondary antibody, and analysed on an Epics PROFILE flow cytometer. E protein was also expressed on the recombinant virus infected cell surface as same as JEV infected cells.

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combinants which had a signal sequence, however the group 7 epitope was better expressed than the group 8 epitope which was the most potent JEV specific protective epitope. The group 8 epitope may be a discontinuous epitope which may be constructed with amino acids at or near the N terminal region and in the middle part of the C terminal half of the E protein.

#### IMMUNOGENICITY OF THE E PROTEIN PRODUCED BY THE RECOMBINANTS

Immunogenicities of the E proteins produced by the recombinant viruses were examined. E protein concentrated from the culture supernatant of the recombinant baculovirus infected cells by ultracentrifugation or the recombinant baculovirus infected cells were injected into mice with complete Freund's adjuvant 2 times. A week after the last inoculation, sera collected from the mice were assayed for neutralizing and HI activity. The E protein expressed by the recombinant baculovirus which contained M and E coding regions of the JEV genome could elicit significant neutralizing and HI antibodies in the mice (Table 2).

Rabbits were vaccinated intradermally with the recombinant vaccinia virus which had prM

and E coding region with signal sequences and the antibodies in the sera were titrated. HI and neutralizing antibody activities against JEV rapidly increased in the serum of the vaccinated rabbits and continued over 3 months. Four week old C3H/He inbred mice were vaccinated with the recombinant vaccinia virus or parent non recombinant virus to analyze induction of protective immunity by vaccination with the recombinant. Two weeks after the vaccination mice were challenged with 300PFU of JEV intravenously. Mice vaccinated with the recombinant virus could escape death by JEV encephalitis completely, ie vaccination with the recombinant vaccinia virus could induce protective immunity in mice (Table 3).

We examined induction of immunologic memory against JEV with the recombinant vaccinia virus in mice. Mice were vaccinated with the recombinant vaccinia viruses which had prM and E, or C, prM, E and NS1 genomes with signal sequences, respectively, by tail scarification or by injection of inactivated JEV vaccine into the intraperitoneal cavity. After 2 weeks, mice were divided into 2 groups. One group of mice were bled and neutralizing antibody in the sera was titrated and another group of mice were boosted by inactivated JEV vaccine in the intraperitoneal cavity. After a week,

Table 2

Antibody responses of mice immunized with the E protein expressed by the recombinant baculovirus.

Immunized with*	Antibody titer**	
	HI	PRNT (log <sub>10</sub> )
JE4 recombinant culture fluid	40	1.98
JE4 recombinant culture cells	40	1.80
Baculovirus culture fluid	< 4	< 0.6
Baculovirus culture cells	< 4	< 0.6
Mouse brain inactivated vaccine	128	2.50

\* JE4 = recombinant baculovirus containing M and E coding sequence of JEV.

\*\* HI = hemagglutination inhibition; PRNT = plaque reduction neutralizing antibody titer.

Table 3

Protection of mice against challenge with JEV after vaccination with recombinant vaccinia virus WRJ6.

Vaccination	No. of mice that survived/no. tested	% Survival	Geometric mean neutralization antibody titer ( $\log_{10}$ ) <sup>*</sup>
None	2/10	20	< 0.6
WR	2/10	20	< 0.6
WRJ6	11/11	100	2.2

WR = wild-type vaccinia virus, WRJ6 = recombinant vaccinia virus containing prM and E coding regions of JEV genome with signal sequence upstream.

\* titer of plaque reduction neutralization antibody on the challenge day.

Table 4

Antibody responses of mice vaccinated with the recombinant vaccinia viruses.

Vaccinated with <sup>*</sup>	Route <sup>**</sup>	Boosted by	Antibody titer PRNT ( $\log_{10}$ ) <sup>***</sup>
WRJ6	Tail	-	1.65
	i.p.	Vaccine	3.57
WRJ10	Tail	-	0.9
	i.p.	Vaccine	3.80
Vaccine	i.p.	-	0.8
	i.p.	Vaccine	2.60

\* WRJ6 = recombinant vaccinia virus containing prM and E genome of JEV with signal sequence upstream, WRJ10 = recombinant vaccinia virus containing C, prM, E and NS1 coding sequences of JEV; inactivated JEV vaccine prepared from infected mouse brain.

\*\* Tail = tail scarification method, i.p. = intraperitoneal cavity.

\*\*\* PRNT = plaque reduction neutralization antibody titer.

neutralizing antibodies in the sera of the boosted mice were titrated. The mice vaccinated with the recombinant viruses produced significant amounts of neutralizing antibody. When the mice were boosted by inactivated JEV vaccine, the production of neutralizing antibody in the mice vaccinated with the recombinants was greatly enhanced. Those titers were 10 times or more higher than that of the mice vaccinated

and boosted with inactivated JEV vaccine alone (Table 4). These results indicate that the recombinant vaccinia viruses can induce strong immunologic memory in mice.

#### REFERENCES

Collett MS, Larson R, Gold C, Strick D, Anderson DK, Purchio AF. Molecular cloning and nucleo-

nia virus

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stream,  
activated

ine alone  
t the re-  
e strong

Anderson  
nd nucleo-

ber 1990

tide sequence of the pestivirus bovine viral  
diarrhea virus. *Virology* 1988; 165 : 191.

Kimura-Kuroda J, Yasui K. Topographical analysis  
of antigenic determinants on envelope glycopro-  
tein V3(E) of Japanese encephalitis virus, using  
monoclonal antibodies. *J Virol* 1983; 45 : 124.

Kimura-Kuroda J, Yasui K. Antigenic comparison of  
envelope protein E between Japanese enceph-  
alitis virus and some other flaviviruses using  
monoclonal antibodies. *J Gen Virol* 1986; 67 :  
2663.

Kimura-Kuroda J, Yasui K. Protection of mice  
against Japanese encephalitis virus by passive  
administration with monoclonal antibodies. *J*  
*Immunol* 1988; 141 : 3606.

Matsuura Y, Miyamoto M, Sato T, Morita C, Yasui  
K. Characterization of Japanese encephalitis  
virus envelope protein expressed by recom-  
binant baculoviruses. *Virology* 1989; 173 :  
674.

Oya A. Japanese encephalitis vaccine. *Acta Paediatr.*

*Jpn* 1988; 30 : 175.

Rice CM, Lencches EM, Eddy SR, Shin SJ, Sheets  
RL, Strause JH. Nucleotide sequence of yellow  
fever virus. *Science* 1985; 229 : 726.

Russell PK, Brandt WE, Dalrymple JM. Chemical  
and antigenic structure of flaviviruses. In :  
Schlesinger (ed), *The Togaviruses*. New York :  
Academic Press, 1980; pp. 503-529.

Sumiyoshi H, Mori C, Fuke I, et al. Complete nu-  
cleotide sequence of the Japanese encephalitis  
virus genome RNA. *Virology* 1987; 161 :  
497.

Westaway EG, Brinton MA, Galdamovich S et al.  
*Flaviviridae. Intervirology* 1985; 24 : 183.

Yasuda A, Kimura-Kuroda J, Ogimoto M, et al.  
Induction of protective immunity in animals  
vaccinated with recombinant vaccinia viruses  
that expressed prM and E glycoproteins of  
Japanese encephalitis virus. *J Virol* 1990; 64 :  
2788.

FIG. 2

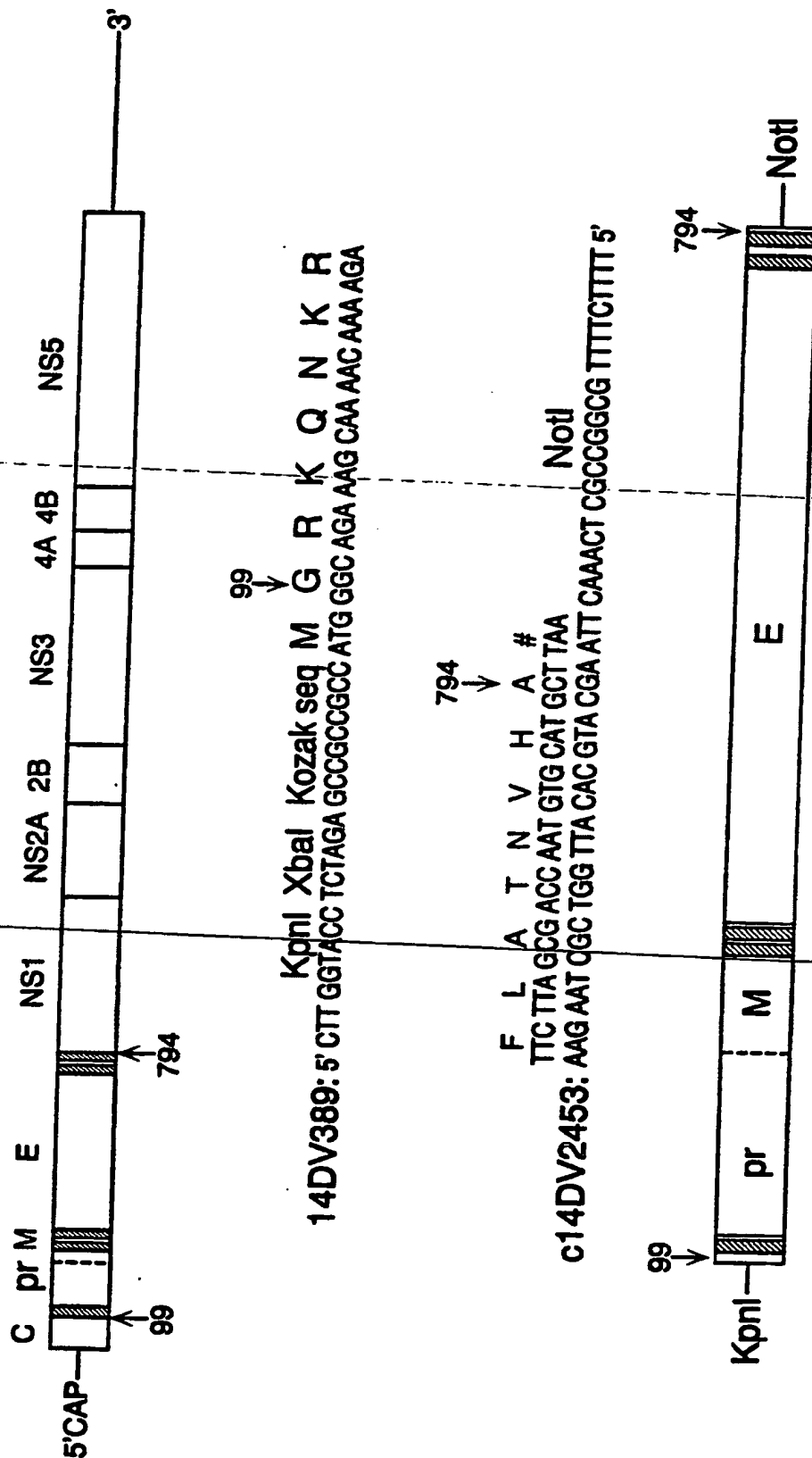


Fig. 2





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(12) **United States Patent**  
**Schmaljohn**

(10) **Patent No.:** **US 6,258,788 B1**  
(45) **Date of Patent:** **Jul. 10, 2001**

(54) **DNA VACCINES AGAINST TICK-BORNE FLAVIVIRUSES**

(75) **Inventor:** **Connie S. Schmaljohn, Frederick, MD (US)**

(73) **Assignee:** **The United States of America as represented by the Secretary of the Army, Washington, DC (US)**

(\*) **Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) **Appl. No.:** **09/197,218**

(22) **Filed:** **Nov. 20, 1998**

#### **Related U.S. Application Data**

(60) **Provisional application No. 60/065,750, filed on Nov. 20, 1997.**

(51) **Int. Cl.<sup>7</sup>** ..... **A61K 39/12**

(52) **U.S. Cl.** ..... **514/44; 424/204.1; 424/218.1**

(58) **Field of Search** ..... **424/204.1, 218.1; 435/6, 5; 514/44**

#### **(56) References Cited**

##### **U.S. PATENT DOCUMENTS**

5,204,253 4/1993 Sanford.  
5,506,125 4/1996 McCabe.

##### **FOREIGN PATENT DOCUMENTS**

0691404 7/1995 (EP).  
9519799 7/1995 (WO).

##### **OTHER PUBLICATIONS**

Colombage, G. et al. (1998) DNA-based and alphavirus-vectored immunisation with prM and E proteins elicits long-lived and protective immunity against the flavivirus, Murray Valley encephalitis virus. *Virology* 250, 151-163.  
Schmaljohn, C. et al. (1997) Naked DNA vaccines expressing the prM and E genes of Russian spring summer encephalitis virus and Central European encephalitis virus protect mice from homologous and heterologous challenge. *J. Virol.* 71, 9563-9568.

Konishi, E. et al. (1992) Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal infection. *Virology* 188, 714-720.

Pletnev, A. G. et al. (1986) Tick-borne encephalitis virus genome: The nucleotide sequence coding for virion structural proteins. *FEBS* 200, 317-321.

Wallner, G. et al. (1996) Characterization and complete genome sequences of high- and low-virulence variants of tick-borne encephalitis virus. *J. Gen. Virol.* 77, 1035-1042.  
Schmaljohn et al., Evaluation of Tick-Borne Encephalitis DNA Vaccine in Monkeys. *Virology* 263:166-174, 1999.

Aberle et al., A DNA Immunization Model Study with Constructs Expressing the Tick-Borne Encephalitis Virus Envelope Protein E in Different Physical Forms. *J. Immunol.* 163(12):6756-6761, 1999.

Kozak, Marilyn; "The Scanning Model for Translation: An Update", *The J. of Cell Biology*, vol. 108, Feb. 1989, pp. 229-241.

Heinz, "Characterization of Tick-Borne Encephalitis Virus and Immunogenicity of its Surface Components in Mice", *Acta Virol.*, 21:308-316 (1977).

Hambleton, et al., "Pathogenesis and Immune Response of Vaccinated and Unvaccinated Rhesus Monkeys to Tick-Borne Encephalitis Virus", *Infection and Immunity*, Jun. 1983, pp 995-1003.

Iacono-Connors, et al., "Characterization of Langat virus antigenic determinants defined by monoclonal antibodies to E, NS1 and prM and identification of a protective, non-neutralizing prM-specific monoclonal antibody", *Virus Research*, 43 (1996), pp. 125-136.

Holzmann, et al., "A Single Amino Acid Substitution in Envelope Protein E of Tick-Borne Encephalitis Virus Leads to Attenuation in the Mouse Model", *J. Virology*, Oct. 1990, pp. 5156-5159.

Schalich, et al., "Recombinant Subviral Particles from Tick-Borne Encephalitis Virus are Fusogenic and Provide a Model System for Studying Flavivirus Envelope Glycoprotein Functions", *J. Virology*, Jul. 1996, pp. 4549-4557.

Konishi, et al., "A Highly Attenuated Host Range-Restricted Vaccinia Virus Strain, NYVAC, Encoding the prM, E, and NS1 Genes of Japanese Encephalitis Virus Prevents JEV Viremia in Swine", *Virology*, 190, (1992) pp. 454-458.

Konishi and Mason, "Proper Maturation of the Japanese Encephalitis Virus Envelope Glycoprotein Requires Cosynthesis with the Premembrane Protein", *J. Virology*, Mar. 1997, pp. 1672-1675.

Kunz et al., "Immunogenicity and Reactogenicity of a Highly Purified Vaccine Against Tick-Borne Encephalitis", *J. Medical Virology*, 6:103-109 (1980).

Holzmann, et al., "Molecular epidemiology of tick-borne encephalitis virus: cross-protection between European and Far Eastern subtypes", *Vaccine*, 10:345-349 (1992).

Pincus, et al., "Recombinant Vaccinia Virus Producing the prM and E Proteins of Yellow Fever Virus Protects Mice from Lethal Yellow Fever Encephalitis", *Virology* 187:290-297 (1992).

Mason et al., "Japanese Encephalitis Virus-Vaccinia Recombinants Produce Particulate Forms of the Structural Membrane Proteins and Induce High Levels of Protection against Lethal JEV Infection", *Virology* 180:294-305 (1991).

Eisenbraun, et al., "Examination of Parameters Affecting the Elicitation of Humoral Immune Responses by Particle Bombardment-Mediated Genetic Immunization", *DNA and Cell Biology*, vol. 12, No. 9, 1993, pp. 791-797.

(List continued on next page.)

**Primary Examiner**—Donna C. Wortman

(74) **Attorney, Agent, or Firm**—Elizabeth Arwine; Charles H. Harris

(57) **ABSTRACT**

Particle mediated immunization of tick-borne flavivirus genes confers homologous and heterologous protection against tick borne encephalitis.

**11 Claims, 8 Drawing Sheets**

- pCMV-IR - x - IT - poly(A) -  
- SUMMERAL PARTICLES

## OTHER PUBLICATIONS

- Fynan et al., "DNA Vaccines: Protective immunizations by parenteral, mucosal, and gene-gun inoculations", *PNAS, USA*, vol. 90, Dec. 1993, pp. 11478-11782.
- Whalen, "DNA Vaccines for Emerging Infectious Diseases: What If?", *Emerging Infectious Diseases*, vol. 2, No. 3, Jul.-Sep., 1996, pp. 168-175.
- Pertmer, et al., "Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA", *Vaccine*, vol. 13, No. 15 (1995), pp. 1427-1430.
- Rambukkana, et al., "In Situ Behavior of Human Langerhans Cells in Skin Organ Culture," *Laboratory Investigation*, vol. 73, No. 4, 1995, pp. 521-531.
- J.D. Bos, "The Skin as an Organ of Immunity", *Clin. Exp. Immunol.*, 107 (1997), (Suppl. 1), pp. 3-5.
- Fuller, et al., "Induction of immunodeficiency virus-specific immune responses in rhesus monkeys following gene gun-mediated DNA vaccination", *J. Medical Primatology*, 25:236-241 (1996).
- Labuda, et al., "Importance of Localized Skin Infection in Tick-Borne Encephalitis Virus Transmission", *Virology*, 219, (1996), pp. 357-366.
- Chu, et al., "Serological Relationships among Viruses in the Hantavirus Genus, Family Bunyaviridae", *Virology* 198, (1994), pp. 196-204.
- Heinz, et al., "A Topological and Functional Model of Epitopes on the Structural Glycoprotein of Tick-Borne Encephalitis Virus Defined by Monoclonal Antibodies", *Virology* 126, (1983), pp. 525-537.
- Heinz, et al., "Recombinant and virion-derived soluble and particulate immunogens for vaccination against tick-borne encephalitis", *Vaccine*, vo. 13, No. 17, (1995), pp. 1636-1642.
- Khozinsky and Semonov, "In Vivo Inhibition by Intact Mouse Serum of the Activity of the Flavivirus-Induced T-Suppressors of Autoreactive T-Lymphocytes", *Acta virol.*, 28:212-217 (1984).
- Gajdosaya and Mayer, "Cell-Mediated Immunity in Flavivirus Infections. I. Induction of Cytotoxic T Lymphocytes in Mice by an Attenuated Virus From the Tick-Borne Encephalitis Complex and its Group-Reactive Character", *Acta Virol.* 25:10-18 (1981).
- Mayer et al., "Transfer with dialysable transfer of T-lymphocyte cytolytic response to tick-borne encephalitis virus antigen in naive mice", *Acta virol.* 24:459-463 (1980).
- Mayer et al., "Dialysable specific transfer factor in mice immunized with attenuated Langat virus from the tick-borne encephalitis complex: generation, action and quantitative assay", *Acta virol.*, 26:453-465 (1982).
- Vargin and Semenov, "Changes of natural killer cell activity in different mouse lines by acute and asymptomatic flavivirus infections", *Acta. virol.* 30:303-308 (1986).
- Lu, et al., "Simian immunodeficiency virus DNA vaccine trial in Macaques", *J. Virology*, vol. 70, No. 6, Jun. 1996, pp. 3978-3991.
- Kenyon et al., "Infection of *Maca radiata* with viruses of the tick-borne encephalitis group," *Microbial Pathogenesis* (1992), 13:399-409.

FIG. 1

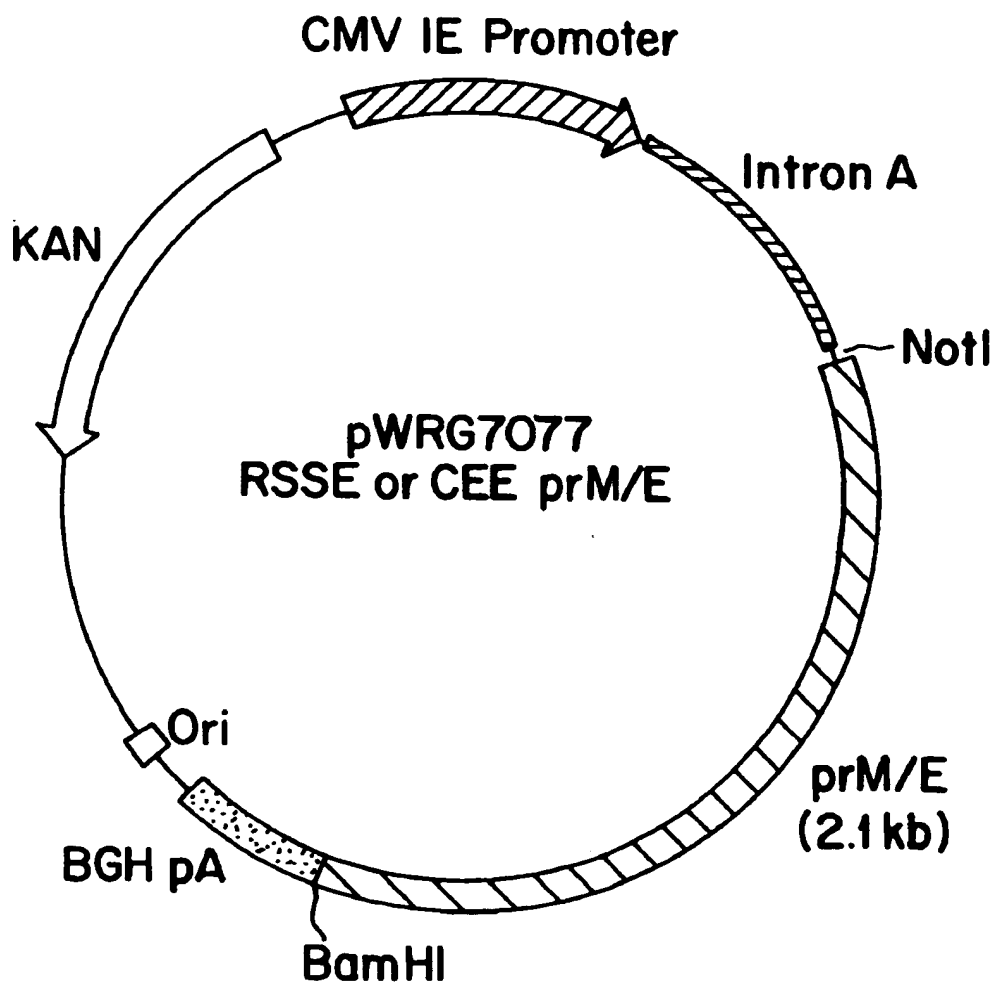


FIG. 2

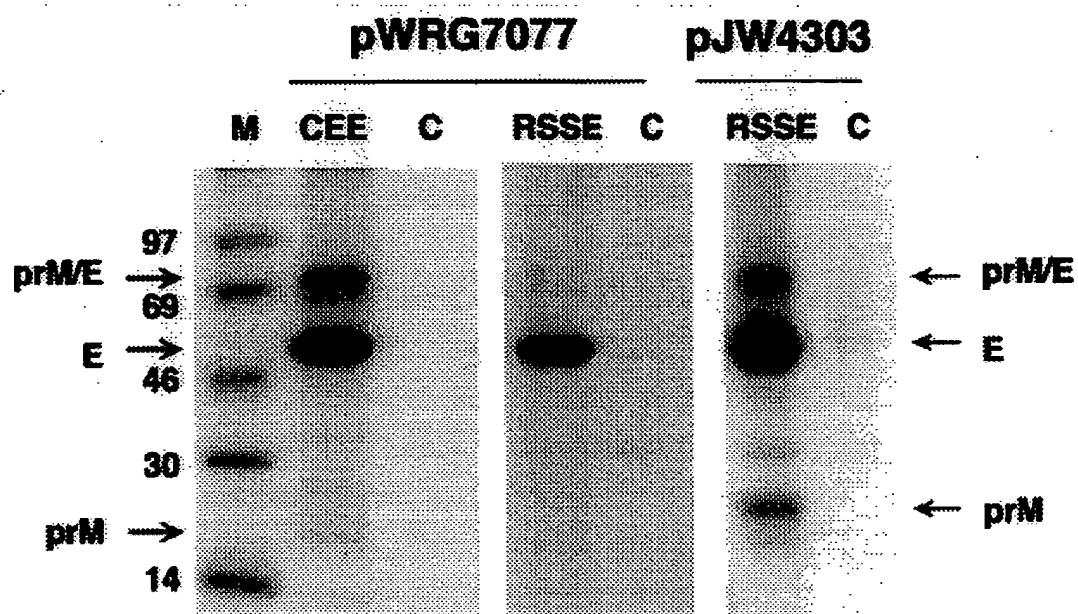


FIG. 3A

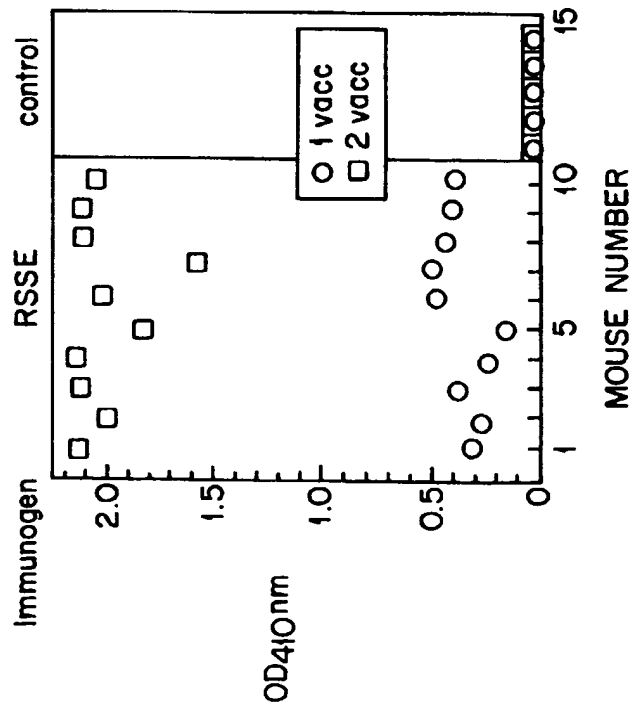


FIG. 3B

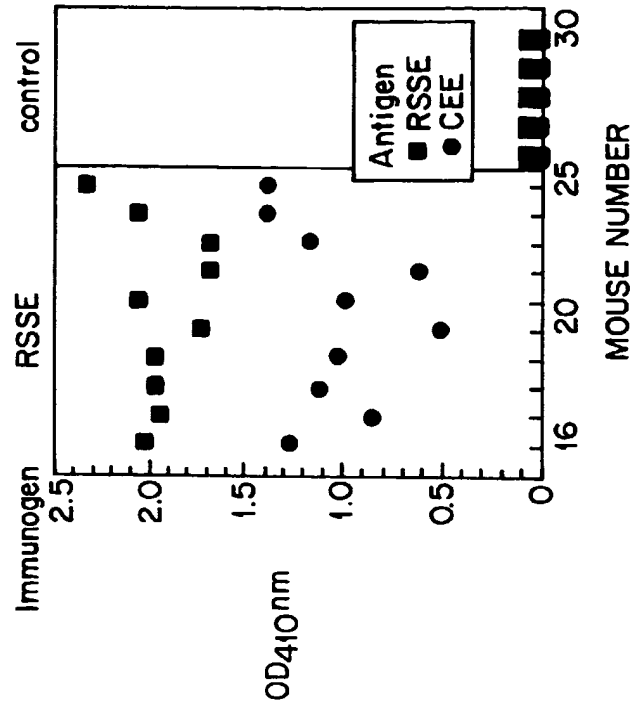


FIG. 4A

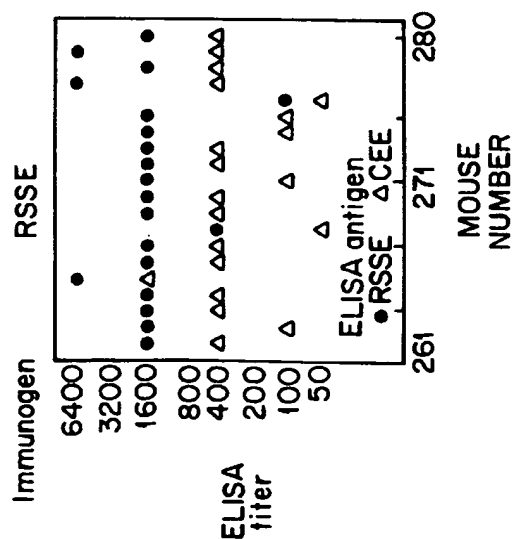


FIG. 4B

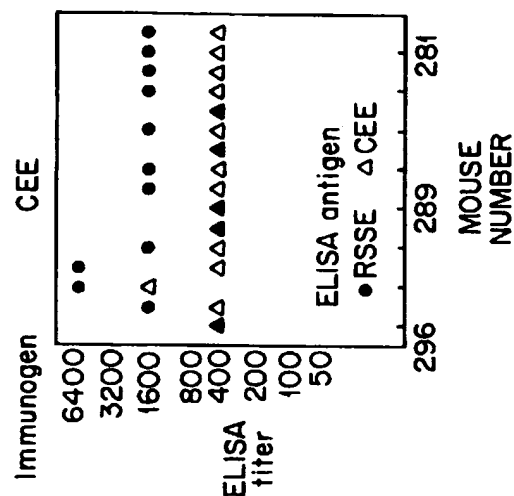
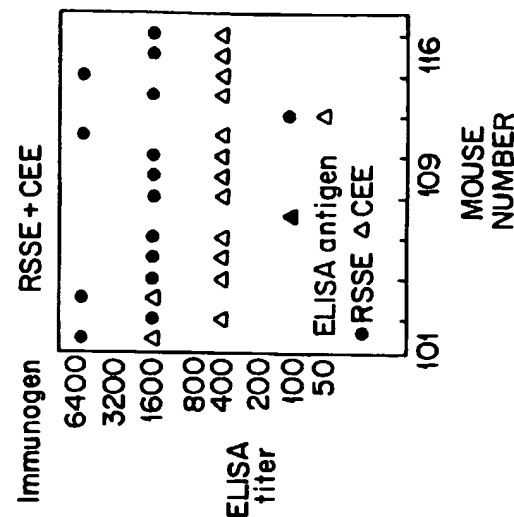


FIG. 4C



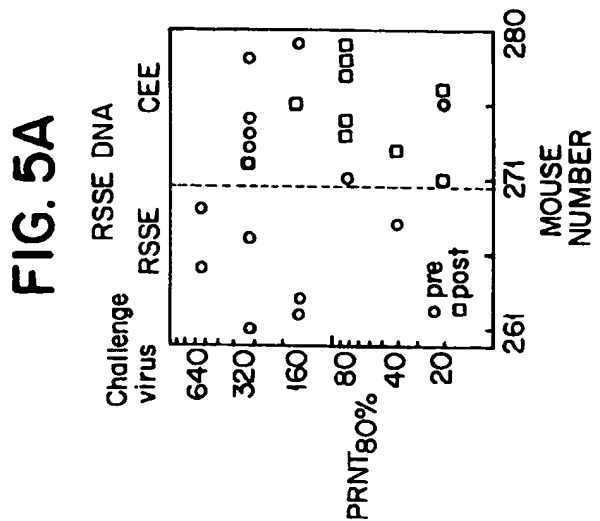
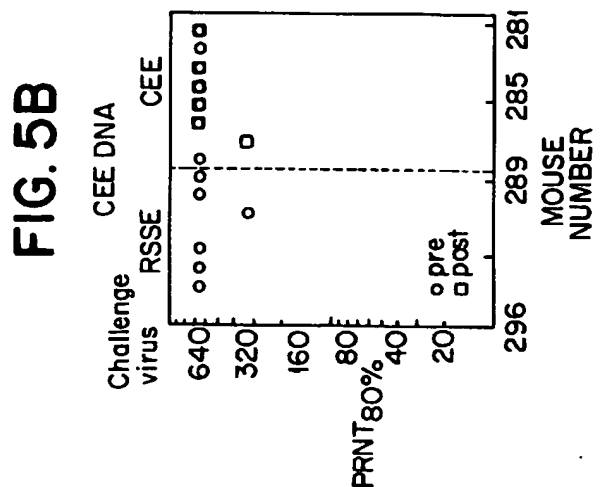
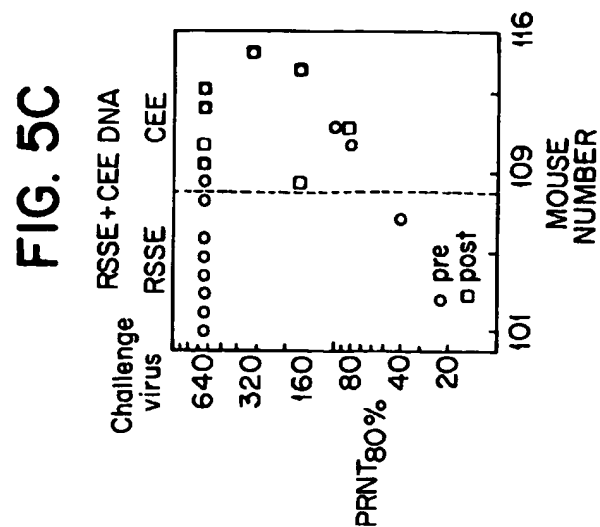


FIG. 6A

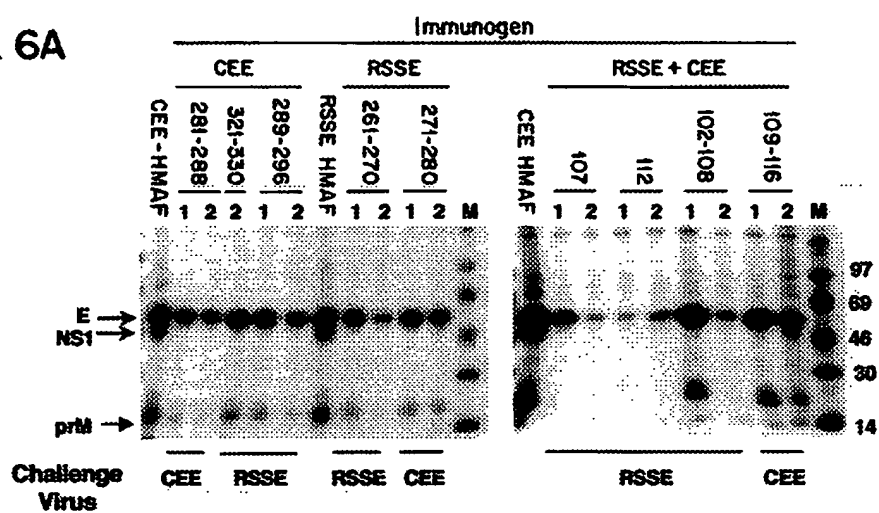


FIG. 6B

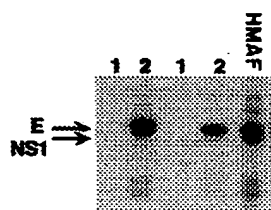


FIG. 6C

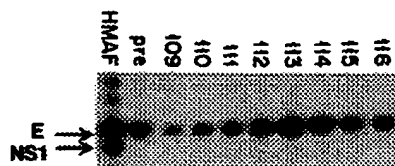




FIG. 7A

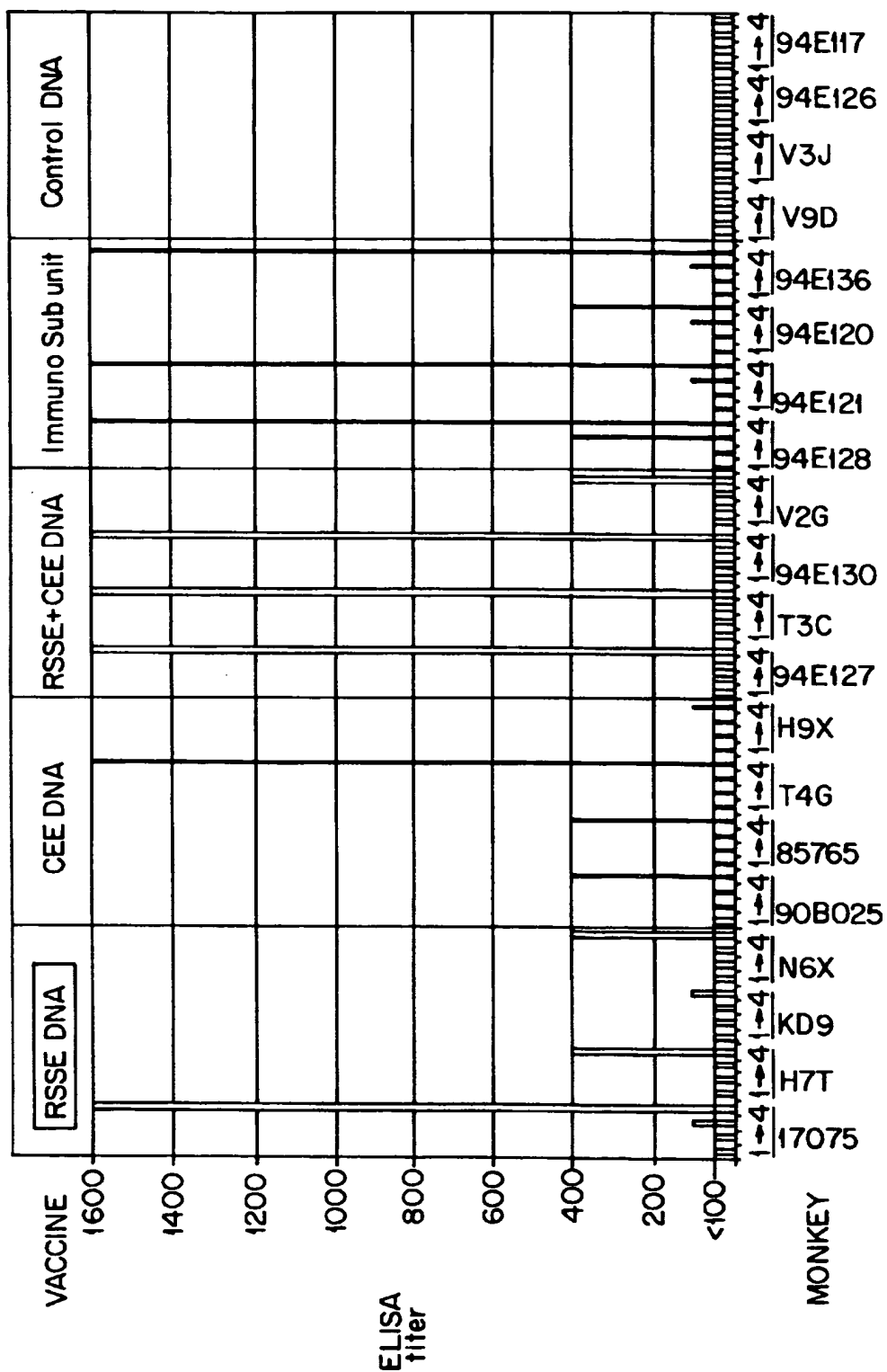
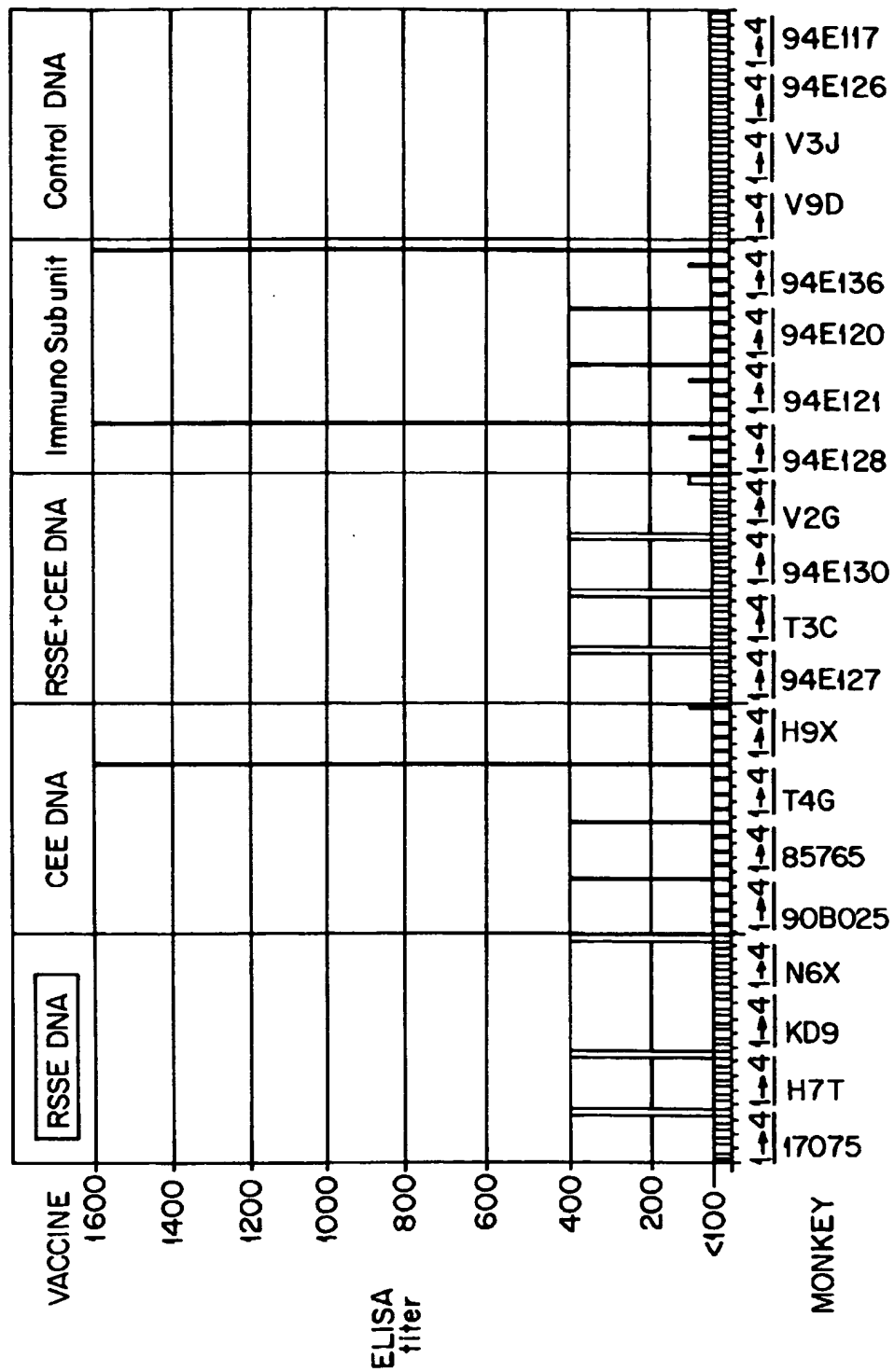


FIG. 7B



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## DNA VACCINES AGAINST TICK-BORNE FLAVIVIRUSES

This application claims the benefit of priority under 35 U.S.C. 119(e) of provisional application Ser. No. 60/065,750 filed on Nov. 20, 1997.

Tick-borne encephalitis (TBE) occurs over a wide area of Europe and the former Soviet Union. TBE is most frequently caused by infection with the flaviviruses Central European encephalitis (CEE) virus, or Russian spring summer encephalitis (RSSE) virus. These viruses are antigenically and genetically closely related to one another and often are considered to be subtypes of the same virus. However, two different tick vectors transmit RSSE and CEE viruses (*Ixodes persulcatus* and *Ixodes ricinus*, respectively) and RSSE virus generally causes a more severe disease than does CEE virus (reviewed in Monath, T. P. and F. X. Heinz, 1996, In B. N. Fields et al. (eds.) *Fields Virology*, Third Edition, Lippincott-Raven Publishers: Philadelphia, p. 961).

In parts of Europe, TBE cases have notably declined since the introduction in 1976 of a formalin-inactivated, chick embryo-derived vaccine. The vaccine is based on an Austrian strain of CEE virus, and elicited protective immunity in mice to the homologous CEE virus (strain Hypr) and to four strains of RSSE virus (Holzmann, H. et al., 1992, *Vaccine*, 10, 345). Despite the success of this vaccine, it suffers the disadvantages commonly associated with inactivated virus vaccines such as the requirement for large-scale production and purification of a highly infectious human pathogen, the risk of incomplete inactivation of the virus, and the need to deliver the vaccine with adjuvant in a three-shot series (Kunz, C. F. et al., 1980, *J. Med. Virol.* 6, 103). Also, this vaccine is not licensed for use in U.S. military personnel nor in U.S. travelers to TBE-endemic regions.

For these reasons, there is a need for an improved TBE vaccine.

### SUMMARY OF THE INVENTION

The present invention satisfies the need discussed above. In this report, we describe two plasmid-based TBE candidate vaccines, which express the premembrane (prM) and envelope (E) genes of RSSE or CEE viruses under control of a cytomegalovirus early promoter. We chose the prM and E genes for expression because of earlier reports with other flaviviruses which indicated that coexpressed prM and E form subviral particles that are able to elicit neutralizing and protective immune responses in animals (Konishi, E. and P. W. Mason, 1993, *J. Virol.* 67: 1672; Konishi, E. et al., 1992, *Virology* 190:454; Pincus, S. et al., 1992, *Virology* 187: 290). Coexpression of prM and E of CEE virus also produces subviral particles, and although these particles were not tested for immunogenicity, they were found to retain biological properties of complete virus such as membrane fusion and hemagglutination (Schalich, J. et al., 1996, *J. Virol.* 70:4549).

To deliver our DNA vaccines, we chose to use the PowderJect-XR™ gene gun device described in WO 95/19799, Jul. 17, 1995. This instrument, which delivers DNA-coated gold beads directly into epidermal cells by high-velocity particle bombardment, was shown to more efficiently induce both humoral and cell-mediated immune responses, with smaller quantities of DNA, than inoculation of the same DNAs by other parenteral routes (Eisenbraun, M. et al., 1993, *DNA Cell. Biol.* 12: 791; Fynan, E. F. et al., 1993, *Proc. Natl. Acad. Sci. U.S.A.* 90: 11478; Haynes, J. R. et al., 1994, *AIDS Res. Hum. Retroviruses* 10: Suppl. 2:S43;

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Pertmer, T. M. et al., 1995, *Vaccine* 13: 1427). Epidermal inoculation of the DNA candidate vaccines also offers the advantages of gene expression in an immunologically active tissue that is generally exfoliated within 15 to 30 days, and which is an important natural focus of viral replication after tick-bite (Bos, J. D., 1997, *Clin. Exp. Immunol.* 107 Suppl. 1:3; Labuda, M. et al., 1996, *Virology* 219:357; Rambukkana, A. et al., 1995, *Lab. Invest.* 73:521; Stingl, G., 1993, *Recent Results Cancer Res.* 128:45). In this application we describe the elicitation of cross-protective immunity to RSSE and CEE viruses by DNA vaccines.

Therefore, the present invention relates to a method for eliciting in an individual an immune response against an alphavirus which causes tick-borne encephalitis comprising delivering to the individual a DNA vaccine comprising a vector including a viral antigen such that when the antigen is introduced into a cell from the individual, the DNA is expressed, the viral antigen is produced in the cell and an immune response against the antigen is mounted.

In one aspect of the invention, the DNA vaccine is delivered by coating a small carrier particle with the DNA vaccine and delivering the DNA-coated particle into an animal's epidermal tissue via particle bombardment. This method may be adapted for delivery to either epidermal or mucosal tissue, or delivery into peripheral blood cells, and thus may be used to induce humoral, cell-mediated, and secretory immune responses in the vaccinated individual.

The DNA vaccine according to the present invention is inherently safe, is not painful to administer, and should not result in adverse side effects to the vaccinated individual. In addition, the invention does not require growth or use of tick-borne flavivirus, which may be spread by aerosol transmission and are typically fatal.

### BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description, appended claims, and accompanying drawings where:

FIG. 1. Schematic of pWRG7077 containing prM and E genes of RSSE and CEE viruses which were amplified by RT-PCR and cloned into NotI and BamHI sites of pWRG7077 (PowderJect Vaccines, Inc., Madison, Wisc.). Characteristics of pWRG7077 are similar to those of pWRG1602 described previously (Dimmock, N. J., 1995, *Med. Virol.* 5: 165) and include a human cytomegalovirus early promoter (CMV IE promoter) and intron A, a bovine growth hormone transcription terminator and polyadenylation signal (BGH pA), and a kanamycin resistance gene.

FIG. 2. Transient expression of naked DNA plasmids in COS cells. Plasmids containing the RSSE or CEE prM and E genes or plasmids with no inserted gene (C) were transfected into COS cells and expression products were immune precipitated with antibodies to RSSE or CEE viruses. Products were analyzed by PAGE and autoradiography. The positions of E, prM and uncleaved prM and E are indicated. The sizes (kD) of molecular weight markers (M) are shown.

FIGS. 3A and B. Antibody responses of mice to naked DNA vaccines as detected by ELISA.

A. Mice were immunized two times, 4 wk apart, with 1 µg/dose of pJW4303 expressing the prM and E genes of RSSE. ELISA was performed on RSSE antigen-coated plates using sera collected just before the second immunization (1 vacc) or 4 wk after the second immunization (2 vacc).

B. Mice were immunized once with 1 µg of pJW4303 expressing the RSSE prM and E genes and, 4 wk later, were

immunized once with 1 µg of pWRG7077 expressing the RSSE prM and E genes. ELISA was performed on RSSE or CEE antigen-coated plates using sera collected 4 wk after the second immunization. Controls for each experiment were comparable plasmids with no gene insert.

FIGS. 4A, B and C. ELISA titers to RSSE and CEE of mice immunized with RSSE (FIG. 4A), CEE (FIG. 4B), or RSSE and CEE (FIG. 4C) DNAs. Mice were immunized three times at 4-wk intervals with 0.5 µg DNA/dose. Titers of sera were determined 4 wk after the final immunization.

FIGS. 5A, B and C. Plaque reduction neutralization by pre- and postchallenge sera of mice immunized with naked DNA vaccines expressing the prM and E genes of RSSE (FIG. 5A), CEE (FIG. 5B) or RSSE and CEE (FIG. 5C) viruses. Twofold dilutions of sera from 1:20 to 1:640 were used in PRNT with CEE virus. PRNT titers are listed as the greatest dilution of serum which resulted in ≥80% reduction of the number of plaques observed in controls incubated with serum from mice vaccinated with control plasmids.

FIGS. 6A–C. Immune precipitation of radiolabeled Langat virus proteins with pre- (lanes 1) and postchallenge (lanes 2) sera from mice vaccinated with naked DNA vaccines expressing the prM and E genes of CEE, RSSE or RSSE and CEE viruses. Immune precipitation products were analyzed by PAGE and autoradiography. Control sera were hyperimmune mouse ascitic fluids (HMAF) to authentic RSSE or CEE viruses. The mouse numbers shown above each autoradiograph correspond to those in FIG. 4 and FIG. 5.

A. Immune precipitation results obtained with pooled sera, except for those labeled 107 and 112, which are individually analyzed serum samples.

B. Immune precipitation results using sera from the two controls that survived challenge with CEE virus.

C. Immune precipitation results from individual sera in group 109–116. The sizes (kD) of molecular weight markers (M) are indicated.

FIGS. 7A and B. Monkey ELISA titers on RSSE (FIG. 7A) and CEE (FIG. 7B) antigen after three immunizations.

### DETAILED DESCRIPTION

In this application is described a composition and method for the vaccination of individuals against tick-borne encephalitis. The method comprises delivery of a DNA encoding an antigen to cells of an individual such that the antigen is expressed in the cell and an immune response is induced in the individual.

DNA vaccination mimicks the de novo antigen production and MHC class I-restricted antigen presentation obtainable with live vaccines, without the risks of pathogenic infection. DNA vaccination involves administering antigen-encoding polynucleotides in vivo to induce the production of a correctly folded antigen(s) within the target cells. The introduction of the DNA vaccine will cause to be expressed within those cells the structural protein determinants associated with the pathogen protein or proteins. The processed structural proteins will be displayed on the cellular surface of the transfected cells in conjunction with the Major Histocompatibility Complex (MHC) antigens of the normal cell. Even when cell-mediated immunity is not the primary means of preventing infection, it is likely important for resolving established infections. Furthermore, the structural proteins released by the expressing transfected cells can also be picked up by antigen-presenting cells to trigger systemic humoral antibody responses.

This vaccine approach is advantageous over subunit vaccines which do not elicit a cytotoxic response necessary to prevent the establishment of infection or disease. Also, this DNA vaccine approach allows delivery to mucosal tissues which may aid in conferring resistance to viral introduction since entry of the virus may be through mucosal tissues.

In order to achieve the immune response sought, a DNA vaccine construct capable of causing transfected cells of the vaccinated individual to express one or more major viral antigenic determinant is necessary. This can be done by identifying regions of the viral genome which code for viral glycoproteins, and joining such coding sequences to promoters capable of expressing the sequences in cells of the vaccinee. Alternatively, the viral genome itself, or parts of the genome, can be used.

In one embodiment, the present invention relates to a DNA or cDNA segment which encodes an antigen from a tick-borne flavivirus such as RSSE, CEE, or Langat. More specifically, prM and E genes of CEE were deduced from the CEE viral genome sequence available in Genbank at accession U39292 (Wallner, G. et al., 1996, *J. Gen. Virol.* 77, 1035–1042) and from RSSE available in Genbank at accession X03870 (Pletnev, A. G. et al., 1986, *FEBS Lett.* 22, 317–321). For CEE, this corresponds to nucleotides 424–2478 (specified in SEQ ID NO:1) of the 10,835 bp genome in Genbank U39292 and the same region was amplified for RSSE, strain Sofjin, but since only a partial gene sequence is available in Genbank, the region amplified corresponds to 419–2470 (specified as SEQ ID NO:2) of the 3,697 bp sequence reported in Genbank X03870. The nucleotide sequences of the amplified regions are about 81% identical. The deduced amino acid sequences are about 94% identical.

DNA or polynucleotide sequences to which the invention also relates include fragments of prM and E containing protective epitopes or antigenic determinants. PrM and E can be delivered in noncontiguous sequences, however, it is preferable that PrM and E be delivered together to get the best results in terms of folding of the proteins and assurance that both proteins are expressed in the same cells.

The derived sequence is not necessarily physically derived from the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO:2, but may be generated in any manner, including for example, chemical synthesis or DNA replication or reverse transcription or transcription, which are based on the information provided by the sequence bases in the region(s) from which the polynucleotide is derived. In addition, combinations of regions corresponding to that of the designated sequence may be modified in ways known in the art to be consistent with an intended use. The sequences of the present invention can be used in diagnostic assays such as hybridization assays and polymerase chain reaction (PCR) assays for the detection of TBE.

The exemplified fragments were obtained using reverse transcription and PCR amplification of a portion of genomic RNA using specific oligonucleotide primers designed to correspond to sequences previously reported for CEE and RSSE viruses (Genbank U39292, X03870, respectively). For the forward primers, nucleotides were modified around the translation initiation codon (bold type below) to generate sequences with a favorable context for translation initiation (Kozak, M., 1989, *J. Cell. Biol.* 108:229). The forward and reverse primers for RSSE were: 5'GCAGTAGACAG-GATGGGTGTTG3' (SEQ ID NO:3) and 5'GCACAGC-CAACTTAAGCTCCCACTCC3' (SEQ ID NO:4). The forward and reverse primers for CEE virus were:

5

5'GCGACGGACAGGATGGGCTGGTTGCTAG3' (SEQ ID NO:5), and 5'CACAGCGCAGCCAACTTACGCCCTCC3' (SEQ ID NO:6). Primers can include additional non-complementary sequences that are fixed during amplification to facilitate subsequent cloning.

It is understood in the art that certain changes to the nucleotide sequence employed in a genetic construct have little or no bearing on the proteins encoded by the construct. Such changes result either from silent point mutations or point mutations that encode different amino acids that do not appreciably alter the behavior of the encoded protein. It is also understood that portions of the coding region can be eliminated without affecting the ability of the construct to achieve the desired effect, namely induction of a protective immune response against tick-borne flavivirus challenge. It is further understood in the art that certain advantageous steps can be taken to increase the antigenicity of an encoded protein by modifying its amino acid composition. Such changes in amino acid composition can be introduced by modifying the genetic sequence encoding the protein. It is contemplated that all such modifications and variations of the tick-borne flavivirus glycoprotein genes are equivalents within the scope of the present invention.

The DNA encoding the desired antigen can be introduced into the cell in any suitable form including, a linearized plasmid, a circular plasmid, a plasmid capable of replication, an episome, RNA, etc. Preferably, the gene is contained in a plasmid. In a particularly preferred embodiment, the plasmid is an expression vector. Individual expression vectors capable of expressing the genetic material can be produced using standard recombinant techniques.

Therefore, in another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The vector can take the form of a plasmid such as pCRII (Invitrogen) or pJW4303 (Konishi, E. et al., 1992, *Virology* 188:714), or any expression vector such as viral vectors e.g. adenovirus or Venezuelan equine encephalitis virus and others known in the art. Preferably, a promoter sequence operable in the target cells is operably linked to the DNA sequence. Several such promoters are known for mammalian systems which may be joined 5', or upstream, of the coding sequence for the encoded protein to be expressed. A suitable promoter is the human cytomegalovirus immediate early promoter. A downstream transcriptional terminator, or polyadenylation sequence, such as the polyA addition sequence of the bovine growth hormone gene, may also be added 3' to the protein coding sequence.

A suitable construct for use in the method of the present invention is pWRG7077 (4326 bp) (PowderJect Vaccines, Inc., Madison, Wisc.), FIG. 1. pWRG7077 includes a human cytomegalovirus (hCMV) immediate early promoter and a bovine growth hormone polyA addition site. Between the promoter and the polyA addition site is Intron A, a sequence that naturally occurs in conjunction with the hCMV IE promoter that has been demonstrated to increase transcription when present on an expression plasmid. Downstream from Intron A, and between Intron A and the polyA addition sequence, are unique cloning sites into which the prME DNA can be cloned. Also provided on pWRG7077 is a gene that confers bacterial host-cell resistance to kanamycin. Any of the fragments that encode RSSE or CEE proteins can be cloned into one of the cloning sites in pWRG7077, using methods known to the art.

In a further embodiment, the present invention relates to host cells stably transformed or transfected with the above-

6

described recombinant DNA construct. The host cell can be prokaryotic such as *Bacillus* or *E. coli*, or eukaryotic such as *Saccharomyces* or *Pichia*, or mammalian cells or insect cells. The vector containing the RSSE or CEE sequence is expressed in the bacteria and the expressed product used for diagnostic procedures or as a vaccine. Please see e.g., Maniatis et al., 1985 *Molecular Cloning: A Laboratory Manual* or *DNA Cloning*, Vol. I and II (D. N. Glover, ed., 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a highly purified IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of RSSE or CEE proteins. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the protein encoded by the DNA. The DNA can be used as circular or linear, or linearized plasmid as long as the CEE and RSSE sequences are operably linked to a promoter which can be expressed in the transfected cell.

In the present invention, the DNA vaccine is transferred into the susceptible individual by means of an accelerated particle gene transfer system. The technique of accelerated particles gene delivery is based on the coating of DNA to be delivered into cells onto extremely small carrier particles, which are designed to be small in relation to the cells sought to be transformed by the process. The DNA sequence containing the desired gene can be simply dried onto a small inert particle. The particle may be made of any inert material such as an inert metal (gold, silver, platinum, tungsten, etc.) or inert plastic (polystyrene, polypropylene, polycarbonate, etc.). Preferably, the particle is made of gold, platinum or tungsten. Most preferably the particle is made of gold. Suitably, the particle is spherical and has a diameter of 0.5 to 5 microns, preferably 1 to 3 microns.

The DNA sequence containing the desired gene prepared in the form suitable for gene introduction can be simply dried onto naked gold or tungsten pellets. However, DNA molecules in such a form may have a relatively short period of stability and may tend to degrade rather rapidly due to chemical reactions with the metallic or oxide substrate of the particle itself. Thus, if the carrier particles are first coated with an encapsulating agent, the DNA strands have greatly improved stability and do not degrade significantly even over a time period of several weeks. A suitable encapsulating agent is polylysine (molecular weight 200,000) which can be applied to the carrier particles before the DNA molecules are applied. Other encapsulating agents, polymeric or otherwise, may also be useful as similar encapsulating agents, including spermidine. The polylysine is applied to the particles by rinsing the gold particles in a solution of 0.02% polylysine and then air drying or heat drying the particles thus coated. Once the metallic particles coated with polylysine were properly dried, DNA strands are then loaded onto the particles.

The DNA is loaded onto the particles at a rate of between 3 and 30 micrograms of DNA per milligram of gold bead spheres. The preferable ratio of DNA to gold is 0.5-5.0 ug of DNA per milligram of gold. A sample procedure begins with gamma irradiated tefzel tubing. The gold is weighed out into a microfuge tube, spermidine (free base) at about 0.05 M is added and mixed, and then the DNA is added. A 10% CaCl solution is incubated along with the DNA for about 10 minutes to provide a fine calcium precipitate. The precipitate carries the DNA with it onto the beads. The tubes are microfuged and the pellet resuspended and washed in 100% ethanol and the final product resuspended in 100%

ethanol at 0.0025 mg/ml PVP. The gold with the DNA is then applied onto the tubing and dried.

The general approach of accelerated particle gene transfection technology is described in U.S. Pat. No. 4,945,050 to Sanford. An instrument based on an improved variant of that approach is available commercially from PowderJect Vaccines, Inc., Madison Wisc., and is described in WO 95/19799. All documents cited herein supra and infra are hereby incorporated in their entirety by reference thereto. Briefly, the DNA-coated particles are deposited onto the interior surface of plastic tubing which is cut to a suitable length to form sample cartridges. A sample cartridge is placed in the path of a compressed gas (e.g., helium at a pressure sufficient to dislodge the particles from the cartridge e.g., 350–400 psi). The particles are entrained in the gas stream and are delivered with sufficient force toward the target tissue to enter the cells of the tissue. Further details are available in the published apparatus application.

The coated carrier particles are physically accelerated toward the cells to be transformed such that the carrier particles lodge in the interior of the target cells. This technique can be used either with cells in vitro or in vivo. At some frequency, the DNA which has been previously coated onto the carrier particles is expressed in the target cells. This gene expression technique has been demonstrated to work in prokaryotes and eukaryotes, from bacteria and yeasts to higher plants and animals. Thus, the accelerated particle method provides a convenient methodology for delivering genes into the cells of a wide variety of tissue types, and offers the capability of delivering those genes to cells in situ and in vivo without any adverse impact or effect on the treated individual. Therefore, the accelerated particle method is also preferred in that it allows a DNA vaccine capable of eliciting an immune response to be directed both to a particular tissue, and to a particular cell layer in a tissue, by varying the delivery site and the force with which the particles are accelerated, respectively. This technique is thus particularly suited for delivery of genes for antigenic proteins into the epidermis.

A DNA vaccine can be delivered in a non-invasive manner to a variety of susceptible tissue types in order to achieve the desired antigenic response in the individual. Most advantageously, the genetic vaccine can be introduced into the epidermis. Such delivery, it has been found, will produce a systemic humoral immune response.

To obtain additional effectiveness from this technique, it may also be desirable that the genes be delivered to a mucosal tissue surface, in order to ensure that mucosal, humoral and cellular immune responses are produced in the vaccinated individual. There are a variety of suitable delivery sites available including any number of sites on the epidermis, peripheral blood cells, i.e. lymphocytes, which could be treated in vitro and placed back into the individual, and a variety of oral, upper respiratory, and genital mucosal surfaces.

Gene gun-based DNA immunization achieves direct, intracellular delivery of DNA, elicits higher levels of protective immunity, and requires approximately three orders of magnitude less DNA than methods employing standard inoculation.

Moreover, gene gun delivery allows for precise control over the level and form of antigen production in a given epidermal site because intracellular DNA delivery can be controlled by systematically varying the number of particles delivered and the amount of DNA per particle. This precise control over the level and form of antigen production may allow for control over the nature of the resultant immune response.

The term transfected is used herein to refer to cells which have incorporated the delivered foreign DNA vaccine, whichever delivery technique is used.

It is herein disclosed that when inducing cellular, humoral, and protective immune responses after DNA vaccination the preferred target cells are epidermal cells, rather than cells of deeper skin layers such as the dermis. Epidermal cells are preferred recipients of DNA vaccines because they are the most accessible cells of the body and may, therefore, be immunized non-invasively. Secondly, in addition to eliciting a humoral immune response, DNA immunized epidermal cells also elicit a cytotoxic immune response that is stronger than that generated in sub-epidermal cells. Delivery to epidermis also has the advantages of being less invasive and delivering to cells which are ultimately sloughed by the body.

Although it can be desirable to induce an immune response by delivering genetic material to a target animal, merely demonstrating an immune response is not necessarily sufficient to confer protective advantage on the animal. What is important is to achieve a protective immune response that manifests itself in a clinical difference. That is, a method is effective only if it reduces the severity of the disease symptoms. It is preferred that the immunization method be at least 20% effective in preventing death in an immunized population after challenge with RSSE or CEE. More preferably, the vaccination method is 50% or more effective, and most preferably 70–100% effective, in preventing death in an immunized population. The vaccination method is shown herein to be 100% effective in the mouse model for TBE. Mice have been used extensively as the laboratory model of choice for assessment of protective immune responses to tick-borne flaviviruses (Gajdosova, E. et al., 1981, *Acta Virol.* 25:10; Heinz, F. X. and C. Kunz, 1982, *J. Biol. Stand.* 10:25; Holzmann, H. et al., 1990, *J. Virol.* 64:5156; Khozinsky, V. V. and B. F. Semenov, 1984, *Acta Virol.* 28:212; Mayer, V. E. et al., 1980, *Acta Virol.* 24:459; Mayer, V. E. et al., 1982, *Acta Virol.* 26:453; Semenov, B. F. et al., 1975, *Med. Biol.* 53:331; Vargin, V. v. and B. F. Semenov, 1986, *Acta Virol.* 30:303; Venugopal, K. et al., 1994, *Res. Vet. Sci.* 57:188). In contrast, unimmunized animals are uniformly killed by challenge with RSSE or CEE. Additionally, either CEE or RSSE immunized mice were able to survive challenge with either CEE or RSSE, indicating homologous and heterologous protection as a result of vaccination. It is expected that cross-protection against other strains of TBE-causing viruses would be achieved (Holzmann, H. et al., 1992, *Vaccine*, 10, 345–349).

Generally, the DNA vaccine administered may be in an amount of about 1–5 ug of DNA per dose and will depend on the subject to be treated, capacity of the subject's immune system to develop the desired immune response, and the degree of protection desired. Precise amounts of the vaccine to be administered may depend on the judgement of the practitioner and may be peculiar to each subject and antigen.

The vaccine may be given in a single dose schedule, or preferably a multiple dose schedule in which a primary course of vaccination may be with 1–10 separate doses, followed by other doses given at subsequent time intervals required to maintain and/or reinforce the immune response, for example, at 1–4 months for a second dose, and if needed, a subsequent dose(s) after several months. Examples of suitable immunization schedules include: (i) 0, 1 months and 6 months, (ii) 0, 7 days and 1 month, (iii) 0 and 1 month, (iv) 0 and 6 months, or other schedules sufficient to elicit the desired immune responses expected to confer protective immunity, or reduce disease symptoms, or reduce severity of disease.

In another embodiment, the present invention provides reagents useful for carrying out the present process. Such reagents comprise a DNA fragment containing prM/E gene from either RSSE or CEE or both RSSE and CEE, and a small, inert, dense particle. The DNA fragment, and dense particle are those described above.

Preferably, the DNA is frozen or lyophilized, and the small, inert, dense particle is in dry powder. If a coating solution is used, the dry ingredients for the coating solution may be premixed and premeasured and contained in a container such as a vial or sealed envelope.

The present invention also provides kits which are useful for carrying out the present invention. The present kits comprise a first container means containing the above-described frozen or lyophilized DNA. The kit also comprises a second container means which contains the coating solution or the premixed, premeasured dry components of the coating solution. The kit also comprises a third container means which contains the small, inert, dense particles in dry powder form or suspended in 100% ethanol. These container means can be made of glass, plastic or foil and can be a vial, bottle, pouch, tube, bag, etc. The kit may also contain written information, such as procedures for carrying out the present invention or analytical information, such as the amount of reagent (e.g. moles or mass of DNA) contained in the first container means. The written information may be on any of the first, second, and/or third container means, and/or a separate sheet included, along with the first, second, and third container means, in a fourth container means. The fourth container means may be, e.g. a box or a bag, and may contain the first, second, and third container means.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

The following materials and method were used in the examples below.

#### MATERIALS AND METHODS

Viruses, cells, media. Viruses were kindly provided by Dr. Robert Shope, Yale Arbovirus Research Unit, New Haven, Conn. Cell lines were obtained from the American Type Culture Collection. Central European encephalitis virus, strain Hypr, was isolated originally in 1953 from a TBE patient in Czechoslovakia. Russian spring summer encephalitis virus, strain Sofjin, was isolated originally in 1937 from a TBE patient from the Far Eastern USSR. Langat virus was isolated originally in 1956 from ticks collected in Malaysia (Calisher, C. H., 1988, *Acta Virol.* 32:469). RSSE and CEE viruses were propagated in VERO E6 cells and Langat virus was propagated in LLC-MK<sub>2</sub> cells. Cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum and antibiotics. Propagation and assay of RSSE or CEE viruses were carried out in a biosafety level 4 laboratory.

Cloning of the prM/E genes of RSSE and CEE. For reverse transcription and polymerase chain reaction (RT-PCR) amplification of the prM and E genes of RSSE and CEE viruses, specific oligonucleotide primers were designed to correspond to sequences previously reported for RSSE and CEE viruses (Genbank U39292, X03870). For the forward primers, nucleotides were modified around the translation initiation codon (bold type below) to generate sequences with a favorable context for translation initiation (Kozak, M., 1989, *J. Cell Biol.* 108:229). The forward and reverse primers for RSSE were: 5'GCAGTAGACAG-

GATGGGTGGTTG3' (SEQ ID NO:3) and 5'GCACAGCAACTTAAGCTCCCACTCC3' (SEQ ID NO:4). The forward and reverse primers for CEE virus were: 5'GCGACGGACAGGATGGGCTGGTTGCTAG3' (SEQ ID NO:5), and 5'CACAGCGCAGCCAACTTACGCCCACTCC3' (SEQ ID NO:6).

Total intracellular RNA of virus-infected Vero cells was extracted by using Trizol reagent (Gibco). For reverse transcription of the RSSE and CEE prM and E genes, the specific oligonucleotide primers, and/or random primers were used with Superscript cDNA synthesis reagents (Gibco). The same specific primers were used to amplify the cDNA by PCR, using Expand HiFi reagents (Boehringer Mannheim). PCR was carried out in a PCR 9600 thermocycler (Perkin Elmer). PCR conditions were 40 cycles of 94° C. for 40 sec, 38° C. for 45 sec, 72° C. for 1 min, after which reactions were incubated at 72° C. for 5 min and then held at 4° C. until used for cloning into the pCRII plasmid (Invitrogen). After verification of orientation, the cDNA inserts were excised from pCRII by digestion with EcoRV and SpeI or by digestion with NotI and partial digestion with BamHI. The RSSE and CEE cDNAs were then cloned into the HindIII (blunt), and NheI sites of pJW4303 (Lu, S. et al., 1996, *J. Virol.* 70:3978) or the NotI and BamHI sites of pWRG7077.

Transient expression assays of RSSE and CEE prM and E genes. For each assay, 5 µg of pWRG7077 containing RSSE or CEE prM and E genes, or control plasmid with no insert, was mixed with 200 µl of OptiMEM medium (Gibco) with no antibiotics. A separate solution was prepared consisting of 40 µl of Lipofectin reagent (Gibco) in 200 µl of OptiMEM (Gibco). Both solutions were incubated at room temperature for 30–45 min, after which they were combined and incubation was continued at room temperature for 10–15 min. OptiMEM (1.6 ml) was then added to each assay and the solution was placed onto monolayers of COS cells, in 25 cm<sup>2</sup> flasks, that had been rinsed one time with 2 ml of serum-free EMEM. The cells were incubated for 7 h at 37° C., then the Lipofectin/DNA solution was removed and fresh OptiMEM with antibiotics was added and incubation was continued. At 26 h postinfection, the medium was removed from the cell cultures and replaced with EMEM without cysteine or methionine. After incubation for 1 h at 37° C., 200 µCi of <sup>35</sup>S Promix (methionine and cysteine, Amersham) was added to each flask and the cells were incubated for 4 h at 37° C. The radiolabeling medium was then removed and cells were lysed on ice with 1 ml of a buffer consisting of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 M NaCl, 4% Zwittergent 3-14 (Calbiochem-Behring) and protease inhibitors (Boehringer Mannheim). Cell nuclei were removed by centrifugation for 5 min at 12,000×g in a microcentrifuge. An aliquot (100 µl) of each supernatant was mixed with 5 µl of a hyperimmune mouse ascitic fluid to RSSE or CEE viruses. After incubation on ice overnight, 100 µl of 50% Protein A Sepharose (Sigma) in lysis buffer was added to each tube, and the samples were shaken at 4° C. for 30 min. The Sepharose beads were recovered by centrifugation in a microcentrifuge and were washed three times with lysis buffer, and one time with 10 mM Tris-HCl, pH 8.0. The beads were then boiled for 2 min in protein sample buffer and analyzed by SDS polyacrylamide gel electrophoresis (PAGE) as described previously (Arikawa, J. et al., 1989, *J. Gen. Virol.* 70:615).

Preparation of gene gun cartridges, immunization and challenge of mice. Plasmid DNA was precipitated onto the outside surface of gold beads (approximately 2 µm in diameter) as described previously (Eisenbraun, M. D. et al.,

1993, supra). The DNA loads were 0.5–1  $\mu\text{g}/\text{mg}$  gold. The DNA-coated gold particles were dried on the inside walls of Tefzel tubing (McMaster Carr), which was then cut into 0.5 inch sections to make cartridges for the gene gun (Pertmer, T. M. et al., 1995, *Vaccine* 13:1427). These cartridges each contained approximately 0.5 mg of gold coated with 0.25–0.5  $\mu\text{g}$  of DNA. BALB/c mice (approximately 6 to 8 wk-old) were immunized by using the hand-held, helium powered PowderJect-XR™ gene gun (Patent WO 95/19799) to deliver approximately 0.5–1  $\mu\text{g}$  of DNA to the epidermis as described in Results and as reported previously (Pertmer, 1995, supra). For challenge studies, mice were transferred to a biosafety level 4 containment area and challenged by intraperitoneal inoculation of approximately 50 PFU of suckling mouse brain-passaged RSSE or CEE virus, a dose previously determined to be approximately 100 LD<sub>50</sub> for BALB/c mice. Mice were observed daily for signs of illness and for death.

ELISA. Direct IgG ELISA was performed by using methods similar to those described previously (Chu, Y.-K. et al., 1994, *Virology* 198:196; Meegan, J. M. et al., 1987, *Am. J. Vet. Res.* 48:1138). The viral antigen was prepared by detergent lysis of RSSE or CEE virus-infected VERO cells and infectious virus was inactivated by gamma irradiation of lysates (Chu, 1994, supra). One half of a 96-well polystyrene (PVC) microtiter plate (Dynatech, Vienna, Va.) was coated directly with 100  $\mu\text{l}$ /well of viral antigen diluted in 0.01 M PBS (pH 7.4) with 0.01% thimerosal (coating buffer) at a predetermined optimal dilution (1:1000). The other half was coated with 100  $\mu\text{l}$ /well of a similarly treated negative antigen made from uninfected cells. Plates were wrapped in plastic wrap and incubated at 4° C. overnight. The next day plates were washed three times with wash buffer (coating buffer and 1% Tween-20; 300  $\mu\text{l}$ /well/wash) by using an automatic plate washer (Biotek Instruments). All subsequent reagents added to the plates were diluted in wash buffer containing 5% skim milk (Difco). After the addition of each reagent, the plates were incubated in a moist environment at 37° C. for 1 h and then washed three times. Serum samples were initially diluted in microtiter tubes (Bio-Rad) and then further diluted from the microtiter tube into both positive and negative coated wells (final dilution of 1:100). Sera were screened at a 1:100 dilution or were serially diluted fourfold from 1:100 to 1:6400 in the ELISA plate. The positive control sera used were ascitic fluids from hyperimmunized mice inoculated with authentic homologous virus. Negative control sera used were prebleeds and controls from mice used in the study. After incubating, plates were washed and 100  $\mu\text{l}$  of horseradish peroxidase (HRPO)-labeled goat anti-mouse IgG antibody (Boehringer Mannheim) (200 ng/ml) was added to each well. The substrate 2,2'-azino-di 3-ethylbenzothiazoline sulfonate (ABTS; Kirkegaard and Perry) was added, and plates were read at 410 nm with a Dynatech MR5000 reader and Lotus Measure. The readings were adjusted by subtracting the optical density (OD) of the negative antigen-coated wells from the positive antigen-coated wells. OD cutoff values were determined as follows: The mean of the adjusted OD values was determined for all the mouse prebleed and control samples and the standard deviation calculated. The cutoff of the assay was the mean OD value plus three standard deviations rounded up to the nearest tenth. An OD value was considered positive if it was greater than or equal to this value. The titer was equal to the reciprocal of the last dilution that was above or equal to the OD cutoff value. A serum sample was considered positive if the titer was  $\geq 1:100$ .

Plaque-reduction neutralization assays (PRNT<sub>80</sub>). Two-fold dilutions of sera (1:20–1:640) were prepared in EMEM supplemented with 10% FBS, and antibiotics. Dilutions were incubated at 56° C. for 30 min to inactivate complement, then were mixed with an equal volume of infectious RSSE or CEE virus in EMEM supplemented with 10% FBS and antibiotics to yield a mixture containing approximately 500 PFU of virus/ml. The virus/antibody mixtures were incubated at 37° C. for 1 h, and then stored at 4° C. overnight. The following day, 0.2 ml of the mixture was added to duplicate wells of six-well plates containing confluent monolayers of VERO E6 cells. The plates were incubated for 1 h (rocking gently every 15–20 min). The wells were then overlaid with 2 ml of 0.6% Seakem ME agarose (FMC Corp.) prepared in EMEM and supplemented with 5% FBS, nonessential amino acids, L-glutamine and antibiotics. The plates were incubated at 37° C. in 5% CO<sub>2</sub> for 6 days, after which a second overlay of 0.5% agarose in EMEM supplemented with 2.5% FBS and neutral red was applied. Plaques were visible 1 to 2 days later. The neutralizing antibody titer was calculated as a reciprocal of the highest dilution, resulting in a 80% reduction of plaques when compared to a control of virus with no added antibody.

Radiolabeling and immune precipitation of Langat virus proteins. Conditions for infection and radiolabeling of Langat virus proteins with <sup>35</sup>S-methionine were described previously (Iacono-Connors, L. et al., 1996, *Virus Res.* 43:125). Briefly, Langat virus-infected LLC-MK<sub>2</sub> cell monolayers in 25 cm<sup>2</sup> flasks were radiolabeled 18–24 h after infection with 200  $\mu\text{Ci}/\text{ml}$  of <sup>35</sup>S-ProMix. The cells were lysed in a buffer consisting of 400 mM NaCl, 50 mM Tris HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.2% deoxycholate, and protease inhibitors. Cell nuclei were removed by centrifugation. Langat virus proteins were immune-precipitated with 2–5  $\mu\text{l}$  of experimental mouse sera and analyzed by SDS-PAGE.

#### EXAMPLE 1

Cloning and transient expression of prM and E genes.

Expression of the prM and E genes of RSSE and CEE were assayed by transfection of plasmids pJW4303 (Lu, S. et al., 1996, *J. Virol.* 70:3978) or pWRG7077 (FIG. 1) containing the RSSE genes, or pWRG7077 containing the CEE genes, into cell cultures. Each of the constructs produced E, prM and uncleaved prM/E which could be immune-precipitated with antibodies to authentic viral proteins (FIG. 2).

#### EXAMPLE 2

Antigenicity of the candidate vaccines.

BALB/c mice were immunized by delivery of DNA-coated gold beads to the abdominal epidermis by particle bombardment with helium pressure using the Accell™ gene gun (Geniva, Madison, Wisc.). For our first experiment and the first immunization of the second experiment, we used RSSE prM/E cloned into pJW4303 (Lu, 1996, supra). For all subsequent studies we used RSSE or CEE prM/E cloned into pWRG7077 (FIG. 1). The two plasmids have the same control elements; i.e., a human cytomegalovirus early promoter and intron A, and a bovine growth hormone polyadenylation/transcription termination signal. However, pWRG7077 does not contain the SV40 virus origin of replication and it has a kanamycin resistance gene rather than an ampicillin resistance gene and is therefore more suitable for the development of human vaccines.

In our initial experiment, 10 mice were immunized with the RSSE construct and five mice were immunized with



13

pJW4303 with no insert. Each mouse received two shots in adjacent sites with a combined total of approximately 1  $\mu$ g of DNA. Four wk after the first immunization, the mice were bled and a second immunization of two shots was given. Four wk after the second immunization, the mice were bled again and sera were assayed by ELISA. All of the mice vaccinated with the RSSE construct had detectable responses to RSSE after one vaccination and all of them had increased responses after the second vaccination (FIG. 3A). None of the serum samples from the control mice displayed any reactivity with RSSE antigen (FIG. 3A).

To assess the ability of the RSSE DNA to elicit an antibody response to CEE virus, we performed a second experiment in which 10 mice were immunized as before with RSSE DNA and five mice were immunized with plasmid with no insert. Four wk after the second vaccination, ELISA was performed using RSSE or CEE antigen. Antibody responses were detected to both antigens with sera from all vaccinated mice (FIG. 3B).

To further evaluate the ability of the RSSE and CEE DNAs to elicit cross-reactive antibody responses, we performed a third experiment, in which we immunized 20 mice with RSSE DNA, 16 mice with CEE DNA, 16 mice with both RSSE and CEE DNA, and 18 mice with plasmid with no insert. As before, two immunizations (each consisting of two gene gun shots) were given at 4-wk intervals, but the DNA dose was reduced from 1  $\mu$ g to 0.5  $\mu$ g at each immunization. The mice were bled 4 wk after the second immunization and serum samples assayed by ELISA. Unexpectedly, we found that although there was an initial response to the antigen, there was not a rise in response after

14

## EXAMPLE 3

## Protective efficacy of the candidate vaccines.

To determine if the DNA vaccines could protect mice from challenge with virulent RSSE and CEE viruses, mice from each of the three experiments described above were challenged either with virulent RSSE or CEE viruses. Some of the mice intended as controls for the RSSE cross-challenge of CEE-vaccinated mice (experiment three, above) were inadvertently vaccinated once with RSSE and CEE DNA. Thus, although all of the vaccinated mice survived challenge with RSSE virus, so did all but one of the controls. Therefore, despite our finding that these mice were clearly immunized, as indicated by their high-titered ELISA and PRNT<sub>80</sub> antibody responses (FIG. 4 and 5), we considered this experiment to be invalid with respect to protection and have not included these findings in the statistical analysis of protection (Table 1). To complete the cross-protection study, we vaccinated a fourth group of 10 mice, two times with 0.5  $\mu$ g of CEE DNA. These mice and nine unvaccinated mice were then challenged with RSSE virus. Of these, all of the controls died, and all of the vaccinated mice survived.

A summary of the results from the series of four challenge experiments are shown in Table 1. All 55 of the mice immunized with plasmids containing the RSSE or CEE genes remained healthy after virus challenge. In contrast, all 27 control mice (18 immunized with plasmid lacking an insert, and nine unimmunized mice) displayed symptoms of infection after virus challenge; 14 of 17 mice died after challenge with RSSE virus, and eight of 10 mice died after challenge with CEE virus.

TABLE 1

Mortality of mice immunized with RSSE, CEE, or RSSE and CEE naked DNA vaccines and challenged with RSSE or CEE viruses								
		No. dead/total no.						
Virus(es) used for Vaccine	Challenge Virus	Replicate 1		Replicate 2		Overall		P*
		Vaccinated	Control	Vaccinated	Control	Vaccinated	Control	
CEE	CEE	0/7	6/8			0/7	6/8	0.006
CEE	RSSE	0/10	9/9			0/10	9/9	0.00001
RSSE	CEE	0/5	2/2	0/10	6/8	0/15	8/10	0.0006
RSSE	RSSE	0/10	2/5	0/10	3/3	0/20	5/8	0.0003
RSSE + CEE	CEE	0/8	6/8			0/8	6/8	0.002

\*Values determined with the test for homogeneity of odds ratios by using the StatXact-Turbo program from Cytel software Corp., Cambridge, MA.

the second immunization (not shown). From other experiments, we knew that 0.5  $\mu$ g of these DNAs were sufficient to elicit antibody responses in mice (not shown). Based on these results and those of other studies (not shown) we determined that a hardware modification to the gene gun (a brass insert which altered the helium flow and was intended to more evenly disperse the gold beads at the target inoculation site) resulted in reduced antigenicity. Consequently, we immunized the mice once more (4 wk after the second immunization) with the RSSE, CEE or RSSE, and CEE DNAs. The mice were then bled, and ELISA titers of sera determined on both RSSE and CEE antigen plates. ELISA with RSSE antigen resulted in antibody titers of 100 to  $\geq$ 6400 (FIG. 4). The CEE antigen used to coat the ELISA plates was apparently not as concentrated as the RSSE antigen, in that titers were uniformly lower with sera from both RSSE and CEE DNA-immunized mice (FIG. 4).

## EXAMPLE 4

## Neutralizing antibody and sterile immunity.

Neutralizing antibodies correlate with protective immunity to tick-borne flaviviruses, as demonstrated in mice by passive transfer of neutralizing monoclonal antibodies to M and E (Heinz, F. X. et al., 1983, *Virology* 126:525; Iacono-Connors, 1996, supra). We measured the neutralizing antibody responses elicited by the vaccines in mice from the third experiment just before challenge. Because we found that CEE virus produced clearer, more easily discernible plaques than did RSSE virus, and because infectious virus assays required biosafety level 4 containment, we performed all PRNT<sub>80</sub> only with CEE virus. We found that all of the mice except one had prechallenge neutralizing antibody titers  $\geq$ 40 (FIG. 5). For samples in which an endpoint titer was reached, postchallenge neutralizing antibody titers were generally the same as or lower than prechallenge titers, suggesting a protection from infection (FIG. 5). For samples

15

with prechallenge titers of  $\geq 640$ , we also assayed pooled sera to estimate an endpoint titer. For the pooled serum samples from the CEE challenge group (109–116), the PRNT<sub>80</sub> titers were 1280 both before and after challenge. These results are also consistent with abortive infection by the challenge virus. The same results were obtained with postchallenge sera from the RSSE challenge group; i.e., the same or lower titers after challenge, but the results are not included in FIG. 5 because of the problem mentioned above with regard to invalid controls.

As another means to measure sterile immunity, we immune-precipitated radiolabeled Langat virus proteins with sera from mice vaccinated with RSSE or CEE DNAs both before and after challenge with RSSE or CEE viruses. Langat virus was used rather than RSSE or CEE viruses for these experiments because we previously demonstrated that Langat proteins are cross-reactive with RSSE and CEE antibodies (Iacono-Connors, 1996, *supra*) and because we were able to use Langat virus at biosafety level 3 containment rather than at level 4. We expected that vaccinated mice would have antibodies to Langat virus E, but not NS1. Postchallenge sera would also display reactivity only with E if the mice were not productively infected with the challenge viruses. If they were infected, however, we expected to also see reactivity with NS1. In addition to pooled samples, individual sera from mice 107 and 112 were assayed as representatives of samples for which a higher postchallenge than prechallenge PRNT titer was observed. Analysis of the immune precipitation products by PAGE revealed that although both E and NS1 proteins were clearly precipitated by polyclonal, hyperimmune mouse ascitic fluids to RSSE or CEE viruses, NS1 was not evident in any of the pre- or postchallenge sera from experimental mice (FIG. 6). The pooled sera from mice 109–116 did have a faint band in the area expected for NS1 (FIG. 6A), so these samples were assayed individually for reactivity with Langat virus proteins. None of these samples immune-precipitated Langat virus NS1 (FIG. 6C). Therefore, the immune-precipitation results and the PRNT results both suggest that sterile immunity was induced in the vaccinated mice.

TABLE 2

Antibody responses (geometric mean titers)* and duration of protective immunity elicited by the CEE DNA/gene gun vaccine administered with varying schedules and dosages					
Group	CEE1	CEE2	CEE3	CEE4	CEE5
# vacc (interval)	1	2 (4 wk)	2 (4 wk)	2 (8 wk)	2 (12 wk)
Challenge	8 wk	8 wk	24 wk	24 wk	24 wk
vacc 1	100	<100	53.6	75.8	nd
(range)	(<100–400)	(<100)	(<100–100)	(<100–100)	
vacc 2		606	162.5	348.2	75.8
(range)		(400–1600)	(<100–400)	(100–1600)	(<100–100)
post	4032	1600	2743	1600	800
(range)	(1600–12800)	(400–6400)	(400–12800)	(400–6400)	(100–1600)
survival <sup>b</sup>	9/9	10/10	9/10	10/10	4/5

\*Geometric mean titers (GMT) for vacc 1 are from blood samples collected immediately before the subsequent vaccination; i.e. 8 wk, 4 wk, 4 wk, or 8 wk after the first vaccination for groups 1–4, respectively. GMT for vacc 2 are from blood samples collected immediately prior to challenge; i.e. 4 wk, 20 wk, 16 wk, or 12 wk after the second vaccination for groups 2–5, respectively.

<sup>b</sup>0/19 unvaccinated controls survived challenge

## EXAMPLE 5

Duration of protective immunity after vaccination.

We examined the length of immunity induced by one or two vaccinations with approximately 0.5  $\mu$ g of DNA given at varying intervals as described in Table 2. Individual sera were analyzed by ELISA and geometric mean titers (GMT) of each group were calculated for samples collected immediately

16

before a subsequent vaccination, or immediately before challenge (Table 2). All mice that received one vaccination or two vaccinations 4 wk apart were protected from challenge at 8 wk after the first vaccination. Two vaccinations, given at 4, 8, or 12-wk intervals protected all but two mice from challenge 6 months after the initial vaccination (Table 2).

## EXAMPLE 6

## 10 Primate studies

We have performed a comprehensive evaluation of DNA vaccines for RSSE and CEE viruses in mice (Schmaljohn, C. S. et al, 1997, *J. Virol.* 71:9563). We demonstrated that high levels of neutralizing antibodies to RSSE and CEE viruses can be elicited with two vaccinations, given 4 weeks apart, with 1 mg of PrM/E DNA. We further demonstrated that a single vaccination protected all mice from challenge 8 weeks later. Two immunizations given at an 8 week interval protected 10/10 mice from challenge 6 months after the first vaccination. Two vaccinations given at a 4 week interval protected 9/10 mice 6 months after the first vaccination, and 10/10 mice challenged 1 year after the initial vaccination. From our studies we conclude that we can achieve both homologous and heterologous protection of mice from challenge with RSSE and CEE viruses with either of the 2 DNA vaccines. Following these successes in mice and prior to human trials, we show that neutralizing antibodies are present following vaccination in rhesus macaques.

RSSE and CEE virus infection of primates: There is no known satisfactory disease model for TBE in monkeys. Studies performed at USAMRIID (Kenyon, R. H. et al., 1992, *Microb. Pathog.* 13:399) in Bonnet monkeys (*Macaca radiata*) demonstrated disease upon infection with some viruses in the TBE complex, but not consistently with RSSE or CEE viruses. Several studies, however, have demonstrated that rhesus macaques (*Macaca mulatta*) are useful antigenicity and immunogenicity models. In one study, rhesus monkeys were used to evaluate the efficacy and safety

60

of a commercial killed vaccine (Hambleton, P. et al., 1983, *Infect. Immun.* 40:995). Vaccinated monkeys exposed intravenously developed a subclinical infection with no histopathological lesions but with transient clinical serum chemistry changes that included elevated aspartate aminotransferase, lactate dehydrogenase, and creatine kinase activities. These chemical abnormalities declined as an immune response developed. The immune response was

65

detected as neutralizing antibody in serum. Antibodies to viral envelope protein and two other infected cell-specific polypeptides were also detected.

To evaluate the immunogenicity of our vaccine, rhesus monkeys were randomized into five groups of four animals each. Group 1 was immunized with the genes that encode prM/E of CEE virus. Group 2 was immunized with RSSE virus prM/E genes. Group 3 animals received both RSSE and CEE prM/E genes. Group 4, the positive control, received the licensed Immuno TBE vaccine. Group 5 animals were vaccinated with the DNA carrier plasmid (WRG7077) without any gene inserts and served as the negative control. DNA vaccinated animals in groups 1, 2, and 5 were immunized with approximately 10 µg of DNA on days 0, 28, and 56. This was administered as four gene gun shots to the skin of the lower abdomen on each of the three immunization dates. Group 3 animals received approximately 20 µg of DNA consisting of four gene gun shots of RSSE and of CEE on each vaccination day. Group 4 received the Immuno vaccine, given at the human dose of 0.5 ml IM in the upper arm on days 0, 18, and 56. Monkeys were bled immediately prior to each vaccination and at day 70 to determine the antibody response by enzyme-linked immunosorbent assay (ELISA). Neutralizing antibody levels were determined by plaque reduction.

After 3 immunizations, sera from monkeys receiving the combination of RSSE and CEE DNA vaccines had ELISA titers (on RSSE and CEE antigens) and neutralizing antibody titers (to CEE virus) equivalent to those elicited by the licensed Austrian vaccine (FIG. 7). Neutralizing antibody levels (PRNT<sub>80</sub>) to CEE virus were less than 1:40 for all prevaccination sera and were >1:1280 for all final bleeds, except for 1 (monkey H71 who had a 1:640 titer). Neutralizing antibody responses to RSSE virus were not measured because all assays with infectious RSSE virus and CEE virus require biosafety level 4 containment and it is technically more difficult to assay RSSE virus using a plaque reduction method than to assay CEE virus.

Because neutralizing antibodies are known to be a correlate of protective immunity, these studies indicate that it is likely that the DNA vaccine will protect humans from tick-borne encephalitis caused by RSSE and CEE.

#### DISCUSSION

The use of nucleic acid vaccines to elicit protective immunity to a variety of viruses has been demonstrated in numerous experimental models (for reviews see Ulmer, J. B. et al., 1996, *Adv. Exp. Med. Biol.* 397:49; Ulmer, J. B. et al., 1995, *Ann. NY Acad. Sci.* 772:117; Ulmer, J. B. et al., 1996, *Curr. Opin. Immunol.* 8:531; Whalen, R. g., 1996, *Emerg. Infect. Dis.* 2:168). In the studies reported here, gene gun administration of microgram quantities of DNA encoding the prM and E genes of RSSE or CEE viruses was effective for inducing homologous and heterologous protective immunity in mice. We designed our candidate vaccines to take advantage of earlier findings that showed that coexpressing prM and E results in the formation of secreted, antigenic and immunogenic subviral particles (Heinz, F. X. et al., 1995, *Vaccine* 13:1636; Konishi, E. and P. W. Mason, 1993, *J. Virol.* 67:1672; Konishi, E. et al., 1992, *Virology* 190:454; Konishi, E. et al., 1992, *Virology* 188:714; Pincus, S. et al., 1992, *Virology* 187:290). Such subviral particles, consisting of heterodimers of prM and E, are also a by product of normal flavivirus morphogenesis; i.e., the so-called "slowly sedimenting hemagglutinins" (SHA) (Heinz, F. and C. Kunz, 1977, *Acta Virol.* 21:308; Mason, P.

W. et al, 1991, *Virology* 180:294; Russell, P. K. et al., 1980, In R. w. Schlesinger (ed.) *The Togaviruses*. Academic Press: New York, p. 503-529). The enhanced immunogenicity of these particles is in part due to the inability of E to assume a native conformation in the absence of prM (Konishi, E. and P. W. Mason, 1993, supra). So, although passively transferred neutralizing monoclonal antibodies to E can protect animals from subsequent flavivirus challenge (Buckley, A. and E. A. Gould, 1985, supra; Gould and Buckley, 1986, supra; Heinz, F. X. et al., 1983, supra; Iacono-Connors et al., 1996, supra; Kaufman, B. M. et al., 1987, supra; Kimura-Kuroda, J. and K. Yasui, 1988, supra; Mason et al., 1989, supra; Mathews and Roehrig, 1984, supra), active immunization with expressed, soluble E is not as efficient as prM and E together for inducing protective immunity (Heinz et al., 1995, supra).

As indicated above, neutralizing antibodies to E are, by themselves, sufficient to protect mice, and presumably humans, from CEE virus. Thus, although DNA vaccines delivered to the epidermis by gene gun inoculation efficiently induce both cell-mediated and humoral immune responses (Haynes et al., 1994, supra; Pertmer et al., 1995, supra), we were most interested in analyzing the induction of neutralizing antibodies as a correlate of protection. Our vaccination strategy of two immunizations of 0.5 to 1 µg of DNA delivered at 4-wk intervals was based on optimal parameters determined for gene gun inoculation of a reporter gene (Eisenbraun et al., 1993, supra). In those studies, it was determined that microgram quantities of DNA were sufficient for maximal protein expression and eliciting antibodies to the expression product. Increasing the amount of DNA from 0.1 to 5 µg of DNA per mg of gold did not result in higher expression levels and it was suggested that the 300 copies of DNA found on a typical gold bead (0.1 µg DNA/mg gold) are all that a single cell can efficiently express (Eisenbraun et al., 1993, supra). Although we did not test lesser amounts of DNA, we did investigate other immunization schedules. Our finding that one vaccination with 0.5 µg of DNA can protect mice for at least 2 months, and two vaccinations can protect for at least 6 months suggests that the immune response generated is long-lived and offers encouragement for further development of this vaccine for human use.

The RSSE and CEE cross-reactive immunity that we observed was not surprising in that the prM and E polypeptide expression products of the two viruses are 94% identical. Nevertheless, it is known that certain E-specific monoclonal antibodies differentiate RSSE and CEE viruses, and that minor changes in E can result in altered neuroinvasiveness in mice (Holzmann et al., 1997, *J. Gen. Virol.* 78:31, supra; Holzmann et al., 1992, *Vaccine* 10:345). Consequently, although either of our DNA vaccines by itself may be sufficient for immunity to TBE-causing flaviviruses, it may be prudent to include both DNAs in a vaccine developed for humans.

In some of our experiments, not only did our candidate vaccines protect mice from death and illness after challenge, but apparently prevented replication of the challenge virus, as indirectly measured by the absence of antibody titer increases and the absence of NS1-specific antibodies after challenge. Of course, neither of these methods is sensitive enough to detect low levels of virus replication, so it is possible that the challenge virus did establish an infection but was quickly eliminated. If sterile immunity did occur, we assume that it was related to neutralization of the challenge virus by circulating antibodies. Among the mechanistic possibilities for this are prevention of adsorption of virus to

host cell receptors, inhibition of fusion of the viral envelope to the host plasma membrane, or alteration of the conformation of the viral envelope proteins to perturb entry of the virus into the host cell (Dimmock, N. J., 1995, *Med. Virol.* 5:165). Whichever mechanism occurred, sterile immunity was apparently not required for protective immunity. This is evidenced by the large increases in antibody titers after challenge of some of the mice in our duration of immunity experiments. Additionally, we show that monkeys receiving the DNA vaccine had ELISA titers on RSSE and CEE antigens and neutralizing antibody titers to CEE virus equivalent to the commercially available inactivated virus vaccine. Since neutralizing antibodies correlate with protective immunity, the DNA vaccine described here is likely to protect humans from tick-borne encephalitis caused by RSSE and CEE.

In conclusion, we feel that the DNA/gene gun technology offers great promise for a new generation of vaccines for TBE. The technology is still new and is undergoing constant modifications and revisions. Nevertheless, gene gun immunization of other DNAs, in quantities similar to those in our studies, effectively induced immune responses in larger animals such as pigs and non-human primates after gene gun inoculation (Fuller, 1995, *supra*; Fuller, D. H. et al., 1996, *J. Med. Primatol.* 25:236). Thus, we expect that the amount of DNA needed for successful vaccination will not present a technical barrier. Also, because gene gun delivery of a candidate virus vaccine for hepatitis B (Geniva) was recently approved for use in a human clinical trial, we anticipate no regulatory obstacles for its eventual use in TBE vaccines for humans.

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What is claimed is:

1. A method for inducing a protective immune response to a tick-borne flavivirus protein in a mammal, comprising

- (i) preparing a nucleic acid encoding an antigenic determinant of a tick-borne flavivirus prM/E protein operatively linked to a CMV promoter operative in cells of a mammal, which nucleic acid is suitable for stably producing the antigenic determinant in a mammal;

- 60 (ii) coating the nucleic acid in (i) onto carrier particles;  
 (iii) accelerating the coated carrier particles into epidermal cells of the mammal in vivo; and  
 (iv) inducing a protective immune response in said mammal upon exposure to a tick-borne flavivirus.  
 2. The method according to claim 1 wherein the carrier particles are gold.

3. The method according to claim 1 wherein the tick-borne flavivirus prM/E protein is selected from the group

27

consisting of Russian spring summer encephalitis prM/E proteins, and Central European encephalitis prM/E proteins.

4. The method according to claim 1 wherein the nucleic acid encodes a protein coding region comprising SEQ ID NO:1.

5. A method for inducing a protective immune response to a tick-borne flavivirus protein in a mammal, comprising

(i) preparing a nucleic acid encoding an antigenic determinant of a Russian spring summer encephalitis tick-borne flavivirus prM/E protein operatively linked to a promoter operative in cells of a mammal, which nucleic acid encodes a protein coding region comprising SEQ ID NO:2 and is suitable for stably producing the antigenic determinant in a mammal;

(ii) coating the nucleic acid in (i) onto carrier particles;

(iii) accelerating the coated carrier particles into epidermal cells of the mammal in vivo; and

(iv) inducing a protective immune response in said mammal upon exposure to a tick-borne flavivirus.

6. The method according to claim 1 wherein the nucleic acid encodes a protein coding region comprising SEQ ID NO: 1 and SEQ ID NO:2.

7. A kit for inducing a protective immune response to a tick-borne flavivirus protein in a mammal, comprising packaged in association:

(a) a nucleic acid encoding an antigenic determinant of a tick-borne flavivirus prM/E protein operatively linked to a CMV promoter operative in cells of a mammal,

28

which nucleic acid is suitable for stably producing the antigenic determinant in a mammal;

(b) one or both of a coating solution and/or components of a coating solution; and

(c) carrier particles.

8. The kit of claim 7, wherein the tick-borne flavivirus prM/E protein is selected from the group consisting of Russian spring summer encephalitis prM/E proteins, and Central European encephalitis prM/E proteins.

9. The kit of claim 7, wherein the nucleic acid encodes a protein coding region comprising SEQ ID NO:1.

10. The kit of claim 7, wherein the nucleic acid encodes a protein coding region comprising SEQ ID NO:1 and SEQ ID NO:2.

11. A kit for inducing a protective immune response to a tick-borne flavivirus protein in a mammal, comprising packaged in association:

(a) a nucleic acid encoding an antigenic determinant of a Russian spring summer encephalitis tick-borne flavivirus prM/E protein operatively linked to a promoter operative in cells of a mammal which nucleic acid encodes a protein coding region comprising SEQ ID NO:2 and is suitable for stably producing the antigenic determinant in a mammal;

(b) one or both of a coating solution and/or components of a coating solution; and

(c) carrier particles.

\* \* \* \* \*



# Naked DNA Vaccines Expressing the prM and E Genes of Russian Spring Summer Encephalitis Virus and Central European Encephalitis Virus Protect Mice from Homologous and Heterologous Challenge

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Naked DNA vaccines expressing the prM and E genes of two tick-borne flaviviruses, Russian spring summer encephalitis (RSSE) virus and Central European encephalitis (CEE) virus were evaluated in mice. The vaccines were administered by particle bombardment of DNA-coated gold beads by *Accell* gene gun inoculation. Two immunizations of 0.5 to 1 µg of RSSE or CEE constructs/dose, delivered at 4-week intervals, elicited cross-reactive antibodies detectable by enzyme-linked immunosorbent assay and high-titer neutralizing antibodies to CEE virus. Cross-challenge experiments demonstrated that either vaccine induced protective immunity to homologous or heterologous RSSE or CEE virus challenge. The absence of antibody titer increases after challenge and the presence of antibodies to E and prM, but not NS1, both before and after challenge suggest that the vaccines prevented productive replication of the challenge virus. One vaccination with 0.5 µg of CEE virus DNA provided protective immunity for at least 2 months, and two vaccinations protected mice from challenge with CEE virus for at least 6 months.

Tick-borne encephalitis (TBE) occurs over a wide area of Europe and the former Soviet Union. TBE is most frequently caused by infection with the flaviviruses Central European encephalitis (CEE) virus and Russian spring summer encephalitis (RSSE) virus. These viruses are antigenically and genetically closely related to one another and often are considered to be subtypes of the same virus. However, two different tick vectors transmit RSSE and CEE viruses (*Ixodes persulcatus* and *Ixodes ricinus*, respectively), and RSSE virus generally causes a more severe disease than does CEE virus (reviewed in reference 33). Also, RSSE and CEE viruses can be distinguished by cross-neutralization (5) and by other serological tests (reviewed in reference 4).

In parts of Europe, TBE cases have notably declined since the introduction in 1976 of a formalin-inactivated, chicken embryo-derived vaccine. The vaccine is based on an Austrian strain of CEE virus and elicited protective immunity in mice to the homologous CEE virus (strain Hypr) and to four strains of RSSE virus (18). Despite the success of this vaccine, it suffers the disadvantages commonly associated with inactivated virus vaccines such as the requirement for large-scale production and purification of a highly infectious human pathogen, the risk of incomplete inactivation of the virus, and the need to deliver the vaccine with adjuvant in a three-shot series (26). Also, this vaccine is not licensed for use in the United States.

For these reasons, we are interested in developing an improved TBE vaccine. In this report, we describe two plasmid-based TBE candidate vaccines which express the premembrane (prM) and envelope (E) genes of RSSE or CEE virus under control of a cytomegalovirus early promoter. We chose

the prM and E genes for expression because of earlier reports with other flaviviruses which indicated that coexpressed prM and E form subviral particles that are able to elicit neutralizing and protective immune responses in animals (22, 24, 35). Co-expression of prM and E of CEE virus also produced subviral particles that retained biological properties of complete virus such as membrane fusion and hemagglutination (39) and which were immunogenic in mice (15).

To deliver our DNA vaccines, we chose to use the *Accell* gene gun (patent application W0 95/197991) (Geniva, Madison, Wis.). This instrument, which delivers DNA-coated gold beads directly into epidermal cells by high-velocity particle bombardment, was shown to more efficiently induce both humoral and cell-mediated immune responses, with smaller quantities of DNA, than inoculation of the same DNAs by other parenteral routes (8, 11, 13, 34). Epidermal inoculation of the DNA candidate vaccines also offers the advantages of gene expression in an immunologically active tissue that is generally exfoliated within 15 to 30 days and which is an important natural focus of viral replication after tick bite (2, 27, 36, 40). The experiments described here were intended to evaluate the elicitation of cross-protective immunity to RSSE and CEE viruses by DNA vaccines.

## MATERIALS AND METHODS

**Viruses, cells, and medium.** Viruses were kindly provided by Robert Shope, Yale Arbovirus Research Unit, New Haven, Conn. Cell lines were obtained from the American Type Culture Collection. CEE virus, strain Hypr, was originally isolated in 1953 from a TBE patient in Czechoslovakia. RSSE virus, strain Sofjin, was originally isolated in 1937 from a TBE patient from the far eastern USSR (4). Langat virus was originally isolated in 1956 from ticks collected in Malaysia. RSSE and CEE viruses were propagated in Vero E6 cells, and Langat virus was propagated in LLC-MK<sub>2</sub> cells. Cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Propagation and assay of RSSE or CEE virus were carried out in a biosafety level 4 laboratory.

**Cloning of the prM and E genes of RSSE and CEE viruses.** For reverse transcription (RT)-PCR amplification of the prM and E genes of RSSE and CEE viruses, specific oligonucleotide primers were designed to correspond to se-

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RSSE/CRR prM/E CMU#R

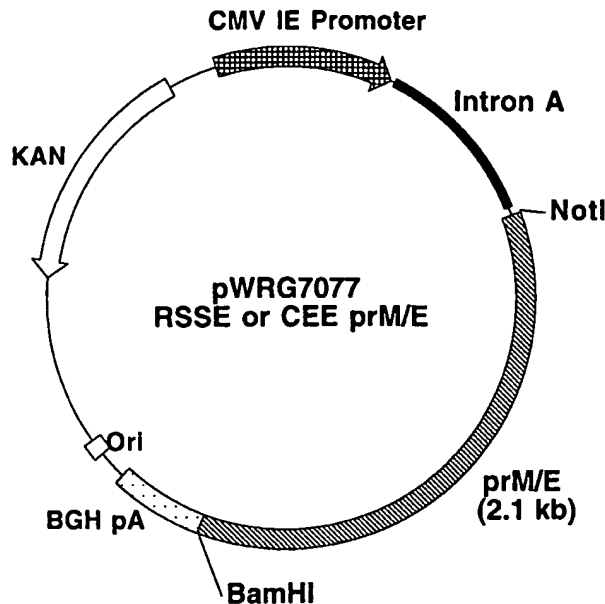


FIG. 1. Schematic of pWRG7077 containing prM and E genes of RSSE and CEE viruses. Genes were amplified by RT-PCR and cloned into *NotI* and *BamHI* sites of pWRG7077. Characteristics of pWRG7077 are similar to those of pWRG1602 described previously (8) and include a human cytomegalovirus immediate-early (CMV IE) promoter, intron A, a bovine growth hormone transcription terminator and polyadenylation signal (BGH pA), and a kanamycin resistance gene.

quences previously reported for RSSE and CEE viruses (Genbank U39292 and X03870, respectively). For the forward primers, nucleotides were modified around the translation initiation codons (bold type below) to generate sequences with a favorable context for translation initiation (25). The forward and reverse primers for RSSE virus were 5'-GCAGTAGACAGGATGGGTTGGTTG3' and 5'-GCACAGCCAACITTAAGTCCCACTCC3', respectively. The forward and reverse primers for CEE virus were 5'-GCGACGGACAGGATGGGCTGGTTGCTAG3' and 5'-CACAGCGCAGCCAACITACGCCCACTCC3', respectively.

Total intracellular RNA of virus-infected Vero cells was extracted by using Trizol reagent (Gibco). For RT of the RSSE and CEE virus prM and E genes, the specific oligonucleotide primers and/or random primers were used with Superscript cDNA synthesis reagents (Gibco). The same specific primers were used to amplify the cDNA by PCR with Expand HiFi reagents (Boehringer Mannheim). PCR was carried out in a PCR 9600 thermocycler (Perkin-Elmer). PCR conditions were 40 cycles of 94°C for 40 s, 38°C for 45 s, and 72°C for 1 min, after which reactions were incubated at 72°C for 5 min and then held at 4°C until used for cloning into the pCR11 (Invitrogen). After verification of orientation, the cDNA inserts were excised from pCR11 by digestion with *EcoRV* and *SpeI* or by digestion with *NorI* and partial digestion with *BamHI*. The RSSE and CEE virus cDNAs were then cloned into the *HindIII* (blunt) and *NheI* sites of pJW4303 (28) or the *NotI* and *BamHI* sites of pWRG7077 (Fig. 1).

**Transient-expression assays of RSSE and CEE virus prM and E genes.** For each assay, 5 µg of pWRG7077 containing RSSE or CEE virus prM and E genes or control plasmid with no insert was mixed with 200 µl of OptiMEM medium (Gibco) with no antibiotics. A separate solution was prepared consisting of 40 µl of Lipofectin reagent (Gibco) in 200 µl of OptiMEM (Gibco). Both solutions were incubated at room temperature for 30 to 45 min, after which they were combined and incubation was continued at room temperature for 10 to 15 min. OptiMEM (1.6 ml) was then added to each assay, and the solution was placed onto monolayers of COS cells in 25-cm<sup>2</sup> flasks that had been rinsed one time with 2 ml of serum-free EMEM. The cells were incubated for 7 h at 37°C, and then the Lipofectin-DNA solution was removed and fresh OptiMEM with antibiotics was added and incubation was continued. At 26-h postinfection, the medium was removed from the cell cultures and replaced with EMEM without cysteine or methionine. After incubation for 1 h at 37°C, 200 µCi of <sup>35</sup>S-labeled Promix (methionine and cysteine; Amersham) was added to each flask and the cells were incubated for 4 h at 37°C. The radiolabeling medium was then removed, and cells were lysed on ice with 1 ml of a buffer consisting of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 M NaCl, 4% Zwittergent 3-14 (Calbiochem-Behring), and protease inhibitors (Boehringer Mannheim). Cell nuclei were removed by centrifugation for 5 min at 12,000 × g in a microcentrifuge. An aliquot (100 µl) of

each supernatant was mixed with 5 µl of a hyperimmune mouse ascitic fluid to RSSE or CEE virus. After incubation on ice overnight, 100 µl of 50% protein A-Sepharose (Sigma) in lysis buffer was added to each tube, and the samples were shaken at 4°C for 30 min. The Sepharose beads were recovered by centrifugation in a microcentrifuge and were washed three times with lysis buffer and one time with 10 mM Tris-HCl, pH 8.0. The beads were then boiled for 2 min in protein sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) as described previously (1).

**Preparation of gene gun cartridges, immunization, and challenge of mice.** Plasmid DNA was precipitated onto the outside surface of gold beads (approximately 2 µm in diameter) as described previously (8). The DNA loads were 0.5 to 1 µg/mg of gold. The DNA-coated gold particles were dried on the inside walls of Tefzel tubing, which was then cut into 0.5-in sections to make cartridges for the gene gun (34). These cartridges each contained approximately 0.5 mg of gold coated with 0.25 to 0.5 µg of DNA. BALB/c mice (approximately 6 to 8 weeks old) were immunized by using the hand-held, helium-powered *Accell* gene gun to deliver approximately 0.5 to 1 µg of DNA to the epidermis as described in Results and as reported previously (34). For challenge studies, mice were transferred to a biosafety level 4 containment area and challenged by intraperitoneal inoculation of approximately 50 PFU of suckling mouse brain-passaged RSSE or CEE virus, a dose previously determined to be approximately 100 times the 50% lethal dose (LD<sub>50</sub>) for BALB/c mice. Mice were observed daily for signs of illness and for death. This research was conducted in accordance with procedures described in the *Guide for the Care and Use of Laboratory Animals* (prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission of Life Sciences-National Research Council) (33a). The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

**ELISA.** Direct immunoglobulin G enzyme-linked immunosorbent assay (ELISA) was performed by using methods similar to those described previously (6, 32). The viral antigen was prepared by detergent lysis of RSSE or CEE virus-infected Vero cells, and infectious virus was inactivated by gamma irradiation of lysates (6). One half of a 96-well polyvinylchloride microtiter plate (Dynatech, Vienna, Va.) was coated directly with 100 µl of viral antigen/well diluted in 0.01 M phosphate-buffered saline (pH 7.4) with 0.01% thimerosal (coating buffer) at a predetermined optimal dilution (1:1,000). The other half was coated with 100 µl/well of a similarly treated negative antigen made from uninfected cells. Plates were wrapped in plastic wrap and incubated at 4°C overnight. The next day plates were washed three times with wash buffer (coating buffer and 1% Tween-20; 300 µl/well/wash) by using an automatic plate washer (Biotek Instruments). All subsequent reagents added to the plates were diluted in wash buffer containing 5% skim milk (Difco). After the addition of each reagent, the plates were incubated in a moist environment at 37°C for 1 h and then washed three times. Serum samples were initially diluted in microtiter tubes (Bio-Rad) and then further diluted from the microtiter tube into wells coated with either positive or negative antigen (final dilution, 1:100). Sera were screened at a 1:100 dilution or were serially diluted fourfold from 1:100 to 1:6,400 in the ELISA plate. The positive-control sera used were ascitic fluids from hyperimmunized mice inoculated with authentic homologous virus. Negative-control sera used were prebleeds and controls from mice used in the study. After incubation, plates were washed and 100 µl of horseradish peroxidase-labeled goat anti-mouse immunoglobulin G antibody (200 ng/ml; Boehringer Mannheim) was added to each well. The substrate 2,2'-azino-di-3-ethylbenzothiazoline sulfonate (ABTS; Kirkegaard and Perry) was added, and plates were read at 410 nm with a Dynatech MR5000 reader and Lotus Measure. The readings were adjusted by subtracting the optical density (OD) of the negative antigen-coated wells from that of the positive antigen-coated wells. OD cutoff values were determined as follows. The mean of the adjusted OD values was determined for all the mouse prebleed and control samples and the standard deviation was calculated. The cutoff of the assay was the mean OD value plus 3 standard deviations rounded up to the nearest tenth. An OD value was considered positive if it was greater than or equal to this value. The titer was equal to the reciprocal of the last dilution that was above or equal to the OD cutoff value. A serum sample was considered positive if the titer was ≥ 1:100.

**PRNT assays.** Twofold dilutions of sera (1:20 to 1:640) were prepared in EMEM supplemented with 10% FBS and antibiotics. Dilutions were incubated at 56°C for 30 min to inactivate complement and then were mixed with an equal volume of infectious RSSE or CEE virus in EMEM supplemented with 10% FBS and antibiotics to yield a mixture containing approximately 500 PFU of virus/ml. The virus-antibody mixtures were incubated at 37°C for 1 h and then stored at 4°C overnight. The following day, 0.2 ml of the mixture was added to duplicate wells of six-well plates containing confluent monolayers of Vero E6 cells. The plates were incubated for 1 h (rocking gently every 15 to 20 min). The wells were then overlaid with 2 ml of 0.6% Seakem ME agarose (FMC Corp.) prepared in EMEM and supplemented with 5% FBS, nonessential amino acids, L-glutamine, and antibiotics. The plates were incubated at 37°C in 5% CO<sub>2</sub> for 6 days, after which a second overlay of 0.5% agarose in EMEM supplemented with 2.5% FBS and neutral red was applied. Plaques were visible 1 to 2 days later. The neutralizing antibody titer was calculated as the reciprocal of the highest dilution resulting in an 80% reduction of plaques (80% plaque reduction neutralization titer [PRNT<sub>80</sub>]) compared to a control of virus with no added antibody.

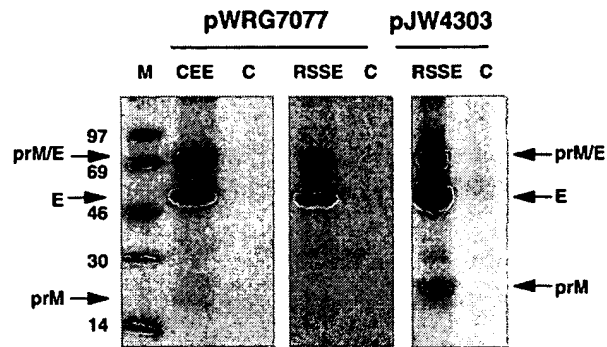


FIG. 2. Transient expression of naked DNA plasmids in COS cells. Plasmids containing the RSSE or CEE virus prM and E genes or plasmids with no inserted gene (C) were transfected into COS cells and expression products were immune precipitated with antibodies to RSSE or CEE virus. Products were analyzed by PAGE and autoradiography. The positions of E, prM, and uncleaved prM and E (prM/E) are indicated. The sizes (kDa) of molecular mass markers (M) are shown on the left.

**Radiolabeling and immune precipitation of Langkat virus proteins.** Conditions for infection and radiolabeling of Langkat virus proteins with [ $^{35}$ S]methionine were described previously (19). Briefly, Langkat virus-infected LLC-MK<sub>2</sub> cell monolayers in 25-cm<sup>2</sup> flasks were radiolabeled 18 to 24 h after infection with 200  $\mu$ Ci of [ $^{35}$ S]-labeled ProMix/ml. The cells were lysed in a buffer consisting of 400 mM NaCl, 50 mM Tris HCl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 0.2% deoxycholate, and protease inhibitors. Cell nuclei were removed by centrifugation. Langkat virus proteins were immune precipitated with 2 to 5  $\mu$ l of experimental mouse sera and analyzed by sodium dodecyl sulfate-PAGE.

## RESULTS

**Cloning and transient expression of prM and E genes.** Expression of the prM and E genes of RSSE and CEE viruses was assayed by transfection of plasmid pJW4303 (28) or pWRG7077 (Fig. 1), containing the RSSE genes, or pWRG7077, containing the CEE genes, into cell cultures. Each of the constructs produced E, prM, and uncleaved prM/E which could be immune precipitated with antibodies to authentic viral proteins (Fig. 2). The cleavage of prM and E is thought to occur by cellular signal peptidase and that of prM and M by the action of another host enzyme, perhaps furin (37).

**Antigenicity of the candidate vaccines.** BALB/c mice were immunized by delivery of DNA-coated gold beads to the abdominal epidermis by particle bombardment with helium pressure by using the *Accell* gene gun. For our first experiment and the first immunization of the second experiment, we used RSSE virus prM-E cloned into pJW4303 (28). For all subsequent studies we used RSSE or CEE virus prM-E cloned into pWRG7077 (Fig. 1). The two plasmids have the same control elements, i.e., a human cytomegalovirus early promoter, intron A, and a bovine growth hormone polyadenylation-transcription termination signal. However, pWRG7077 does not contain the simian virus 40 origin of replication and it has a kanamycin resistance gene rather than an ampicillin resistance gene and is therefore more suitable for the development of human vaccines.

In our initial experiment, 10 mice were immunized with the RSSE virus construct and 5 mice were immunized with pJW4303 with no insert. Each mouse received two shots in adjacent sites with a combined total of approximately 1  $\mu$ g of DNA. Four weeks after the first immunization, the mice were bled and a second immunization of two shots was given. Four weeks after the second immunization, the mice were bled again and sera were assayed by ELISA. All of the mice vaccinated with the RSSE virus construct had detectable responses to

RSSE virus after one vaccination and all of them had increased responses after the second vaccination (Fig. 3A). None of the serum samples from the control mice displayed any reactivity with RSSE virus antigen (Fig. 3A).

To assess the ability of the RSSE virus DNA to elicit an antibody response to CEE virus, we performed a second experiment in which 10 mice were immunized as before with RSSE virus DNA and 5 mice were immunized with plasmid with no insert. Four weeks after the second vaccination, an ELISA was performed with RSSE or CEE virus antigen. Antibody responses were detected to both antigens with sera from all vaccinated mice (Fig. 3B).

To further evaluate the ability of the RSSE and CEE virus DNAs to elicit cross-reactive antibody responses, we performed a third experiment, in which we immunized 20 mice with RSSE virus DNA, 16 mice with CEE virus DNA, 16 mice with both RSSE and CEE virus DNA, and 18 mice with plasmid with no insert. As before, two immunizations (each consisting of two gene gun shots) were given at 4-week intervals, but the DNA dose was reduced from 1  $\mu$ g to 0.5  $\mu$ g at each immunization. The mice were bled 4 weeks after the second immunization, and serum samples were assayed by ELISA. Unexpectedly, we found that although there was an initial response to the antigen, there was not a rise in response after the second immunization (not shown). From other experiments, we knew that 0.5  $\mu$ g of these DNAs was sufficient to elicit antibody responses in mice (not shown). Based on these results and those of other studies (not shown) we determined that a hardware modification to the gene gun (a brass insert which altered the helium flow and was intended to more evenly disperse the gold beads at the target inoculation site) resulted in reduced antigenicity. Consequently, we immunized the mice once more (4 weeks after the second immunization) with the RSSE, CEE and RSSE, or CEE virus DNAs. The mice were then bled, and ELISA titers of sera were determined for both RSSE and CEE virus antigen-coated plates (Fig. 4). As in earlier experiments, increases in ELISA titers were observed in the majority of the samples after this final immunization. The CEE virus antigen used to coat the ELISA plates was apparently not as concentrated as the RSSE virus antigen in that

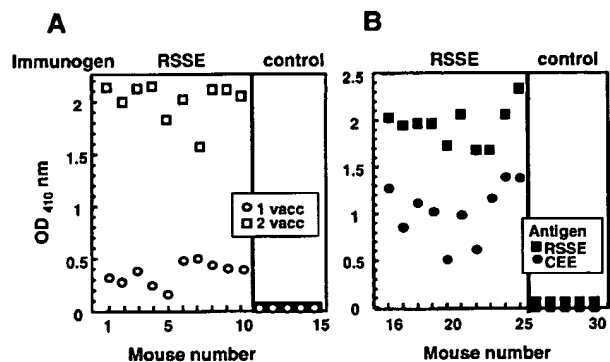


FIG. 3. Antibody responses of mice to naked DNA vaccines as detected by ELISA. (A) Mice were immunized two times, 4 weeks apart, with 1  $\mu$ g of pJW4303/dose expressing the prM and E genes of RSSE virus. ELISA of RSSE virus antigen-coated plates containing sera collected just before the second immunization (1 vacc) or 4 weeks after the second immunization (2 vacc) was performed. (B) Mice were immunized once with 1  $\mu$ g of pJW4303 expressing the RSSE virus prM and E genes, and 4 weeks later, were immunized once with 1  $\mu$ g of pWRG7077 expressing the RSSE virus prM and E genes. ELISA of RSSE or CEE virus antigen-coated plates containing sera collected 4 weeks after the second immunization was performed. Controls for each experiment were comparable plasmids with no gene insert.

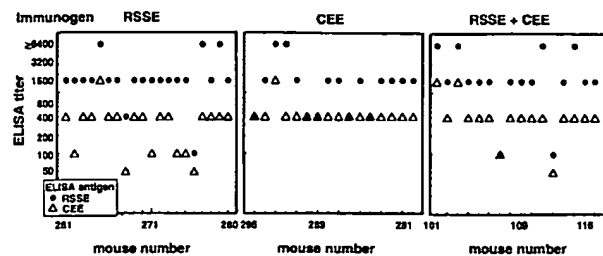


FIG. 4. ELISA titers to RSSE and CEE viruses of mice immunized with RSSE, CEE, or RSSE and CEE virus DNAs. Mice were immunized three times at 4-week intervals with 0.5  $\mu$ g of DNA/dose. Titers of sera were determined 4 weeks after the final immunization.

titers were uniformly lower with sera from both RSSE and CEE virus DNA-immunized mice (Fig. 4).

**Protective efficacy of the candidate vaccines.** To determine if the DNA vaccines could protect mice from challenge with virulent RSSE and CEE viruses, we challenged mice from various experiments with either virulent RSSE or virulent CEE virus. A summary of the challenge results from four experiments is shown in Table 1. All 55 of the mice immunized with plasmids containing the RSSE or CEE virus genes remained healthy after virus challenge. In contrast, all 27 control mice (18 immunized with plasmid lacking an insert and 9 nonimmunized mice) displayed symptoms of infection after virus challenge; 14 of 17 mice died after challenge with RSSE virus, and 8 of 10 mice died after challenge with CEE virus.

**Neutralizing antibody and sterile immunity.** Neutralizing antibodies correlate with protective immunity to tick-borne flaviviruses, as has been demonstrated in mice by passive transfer of neutralizing monoclonal antibodies to M and E (16, 19). We measured the neutralizing antibody responses elicited by the vaccines in mice from the third experiment just before challenge. Because we found that CEE virus produced clearer, more easily discernible plaques than did RSSE virus, and because infectious virus assays required biosafety level 4 containment, we performed all PRNT<sub>80</sub> assays only with CEE virus. PRNT<sub>80</sub>s to CEE virus were higher for mice immunized with CEE virus DNA or with RSSE and CEE virus DNA than for those immunized only with RSSE virus DNA. Such results are consistent with previous studies which differentiated RSSE virus and CEE virus on the basis of cross-neutralization with polyclonal sera (5). We found that all of the mice except one had prechallenge neutralizing antibody titers of  $\geq 40$  (Fig. 5). For samples in which an endpoint titer was reached, postchallenge neutralizing antibody titers were generally the same as or lower than prechallenge titers, suggesting a protection from infection (Fig. 5). For samples with prechallenge titers of

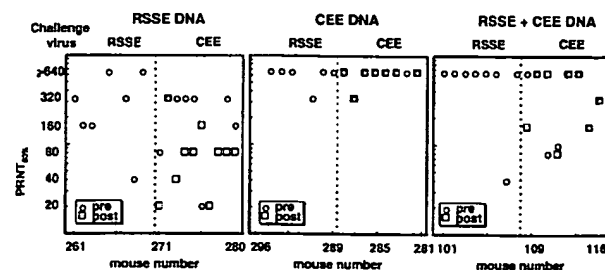


FIG. 5. Plaque reduction neutralization by pre- and postchallenge sera of mice immunized with naked DNA vaccines expressing the prM and E genes of RSSE, CEE, or RSSE and CEE viruses. Twofold dilutions of sera from 1:20 to 1:640 were used in PRNT assays with CEE virus. PRNTs are listed as the greatest dilution of serum which resulted in  $\geq 80\%$  reduction of the number of plaques observed in controls incubated with serum from mice vaccinated with control plasmids.

$\geq 640$ , we also assayed pooled sera to estimate an endpoint titer. For the pooled serum samples from the CEE virus challenge group (mice 109 to 116), the PRNT<sub>80</sub>s were 1,280 both before and after challenge. These results are also consistent with abortive infection by the challenge virus. The same results were obtained with postchallenge sera from the RSSE virus challenge group, i.e., the same or lower titers after challenge, but the results are not included in Fig. 5 because of the problem mentioned above with regard to invalid controls.

As another means to measure sterile immunity, we immune precipitated radiolabeled Langat virus proteins with sera from mice vaccinated with RSSE or CEE virus DNA both before and after challenge with RSSE or CEE virus. Langat virus was used rather than RSSE or CEE virus for these experiments because we previously demonstrated that Langat proteins are cross-reactive with RSSE and CEE virus antibodies (19) and because we were able to use Langat virus at biosafety level 3 containment rather than at level 4. We expected that vaccinated mice would have antibodies to Langat virus E but not NS1. Postchallenge sera would also display reactivity only with E if the mice were not productively infected with the challenge viruses. If they were infected, however, we expected to also see reactivity with NS1. In addition to pooled samples, individual sera from mice 107 and 112 were assayed as representatives of samples for which a higher postchallenge than prechallenge PRNT was observed. Analysis of the immune precipitation products by PAGE revealed that although both E and NS1 proteins were clearly precipitated by polyclonal hyperimmune mouse ascitic fluids to RSSE or CEE virus, NS1 was not evident in any of the pre- or postchallenge sera from experimental mice (Fig. 6). The pooled sera from mice 109 to 116 did have a faint band in the area expected for NS1 (Fig. 6A), so these

TABLE 1. Mortality of mice immunized with RSSE, CEE, or RSSE and CEE virus naked DNA vaccines and challenged with RSSE or CEE virus

Virus(es) used for vaccine	Challenge virus	No. dead/total no.						<i>P</i> <sup>a</sup>
		Replicate 1		Replicate 2		Overall		
		Vaccinated	Control	Vaccinated	Control	Vaccinated	Control	
CEE	CEE	0/7	6/8			0/7	6/8	0.006
CEE	RSSE	0/10	9/9			0/10	9/9	0.00001
RSSE	CEE	0/5	2/2	0/10	6/8	0/15	8/10	0.0006
RSSE	RSSE	0/10	2/5	0/10	3/3	0/20	5/8	0.0003
RSSE + CEE	CEE	0/8	6/8			0/8	6/8	0.002

<sup>a</sup> Values determined with the test for homogeneity of odds ratios by using the StatXact-Turbo program from Cytel Software Corp., Cambridge, Mass.

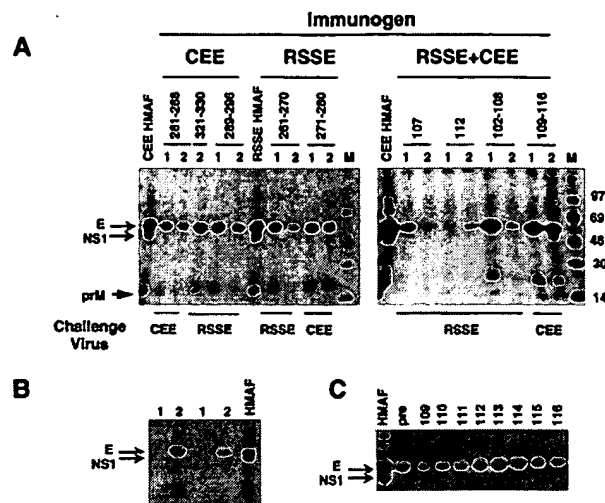


FIG. 6. Immune precipitation of radiolabeled Langkat virus proteins with pre- (lanes 1) and postchallenge (lanes 2) sera from mice vaccinated with naked DNA vaccines expressing the prM and E genes of CEE, RSSE, or RSSE and CEE viruses. Immune precipitation products were analyzed by PAGE and autoradiography. Control sera were hyperimmune mouse ascitic fluids (HMAF) to authentic RSSE or CEE virus. The mouse numbers shown above each autoradiograph correspond to those in Fig. 4 and 5. (A) Immune precipitation results obtained with pooled sera, except for those labeled 107 and 112, which are individually analyzed serum samples. (B) Immune precipitation results for sera from the two controls that survived challenge with CEE virus. (C) Immune precipitation results from individual serum samples in group 109 to 116. The sizes (kDa) of molecular mass markers (M) are indicated on the right of panel A.

samples were assayed individually for reactivity with Langkat virus proteins. None of these samples immune precipitated Langkat virus NS1 (Fig. 6C). Therefore, the immune precipitation results and the PRNT results both suggest that sterile immunity was induced in the vaccinated mice.

**Duration of protective immunity after vaccination.** We examined the length of immunity induced by one or two vaccinations with approximately 0.5  $\mu$ g of DNA given at various intervals as described in Table 2. Individual serum samples were analyzed by ELISA, and geometric mean titers of each group were calculated for samples collected immediately before a subsequent vaccination or immediately before challenge (Table 2). All mice that received one vaccination or two vaccinations 4 weeks apart were protected from challenge at 8 weeks after the first vaccination. Two vaccinations, given at 4-, 8-, or 12-week intervals protected all but two mice from challenge 6 months after the initial vaccination (Table 2).

## DISCUSSION

The use of nucleic acid vaccines to elicit protective immunity to a variety of viruses has been demonstrated in numerous experimental models (for reviews see references 41–44). In the studies reported here, gene gun administration of microgram quantities of DNA encoding the prM and E genes of RSSE or CEE virus was effective for inducing homologous and heterologous protective immunity in mice. We designed our candidate vaccines to take advantage of earlier findings that showed that coexpressing prM and E results in the formation of secreted antigenic and immunogenic subviral particles (15, 22–24, 35). Such subviral particles, consisting of heterodimers of prM and E, are also a byproduct of normal flavivirus morphogenesis, i.e., the so-called slowly sedimenting hemagglutinins (14, 30, 38). The enhanced immunogenicity of these particles is in part due to the inability of E to assume a native conformation in the absence of prM (22). So, although passively transferred neutralizing monoclonal antibodies to E can protect animals from subsequent flavivirus challenge (3, 12, 16, 19–21, 29, 31), active immunization with expressed, soluble E is not as efficient as prM and E together for inducing protective immunity (15).

As indicated above, neutralizing antibodies to E are, by themselves, sufficient to protect mice, and presumably humans, from CEE virus. Thus, although DNA vaccines delivered to the epidermis by gene gun inoculation efficiently induce both cell-mediated and humoral immune responses (13, 34), we were most interested in analyzing the induction of neutralizing antibodies as a correlate of protection. Our vaccination strategy of two immunizations of 0.5 to 1  $\mu$ g of DNA delivered at 4-week intervals was based on optimal parameters determined for gene gun inoculation of a reporter gene (8). In those studies, it was determined that microgram quantities of DNA were sufficient for maximal protein expression and the elicitation of antibodies to the expression product. Increasing the amount of DNA from 0.1 to 5  $\mu$ g of DNA per mg of gold did not result in higher expression levels, and it was suggested that the 300 copies of DNA found on a typical gold bead (0.1  $\mu$ g DNA/mg of gold) are all that a single cell can efficiently express (8). Although we did not test lesser amounts of DNA, we did investigate other immunization schedules. Our finding that one vaccination with 0.5  $\mu$ g of DNA can protect mice for at least 2 months and two vaccinations can protect for at least 6 months suggests that the immune response generated is long-lived and offers encouragement for further development of this vaccine for human use.

Interestingly, although all of our challenge studies were performed with 100 LD<sub>50</sub> of RSSE or CEE virus, some of our control mice that were vaccinated with the plasmid with no

TABLE 2. Antibody responses and duration of protective immunity elicited by CEE virus DNA vaccine<sup>a</sup>

Group	No. of vaccinations (interval [wk])	Wk of challenge	Antibody GMT (range) <sup>b</sup>			No. survived/ no. in group <sup>c</sup>
			1st vaccination <sup>d</sup>	2nd vaccination <sup>d</sup>	Postchallenge	
CEE1	1	8	100 (<100–400)		4,032 (1,600–12,800)	9/9
CEE2	2 (4)	8	<100 (<100)	606 (400–1,600)	1,600 (400–6,400)	10/10
CEE3	2 (4)	24	53.6 (<100–100)	162.5 (<100–400)	2,743 (400–12,800)	9/10
CEE4	2 (8)	24	75.8 (<100–100)	348.2 (100–1,600)	1,600 (400–6,400)	10/10
CEE5	2 (12)	24	ND <sup>e</sup>	75.8 (<100–100)	800 (100–1,600)	4/5

<sup>a</sup> Vaccine administered with gene gun. See Materials and Methods for details.

<sup>b</sup> GMT, geometric mean titer.

<sup>c</sup> Blood samples for groups CEE1 to -4 were collected 8, 4, 4, and 8 weeks after vaccination, respectively.

<sup>d</sup> Blood samples for groups CEE2 to -5 were collected 4, 20, 16, and 12 weeks after the second vaccination, respectively.

<sup>e</sup> ND, not determined.

<sup>f</sup> 0/19 unvaccinated controls survived challenge.

insert did not die (although they did display symptoms of infection). In contrast, all of our unvaccinated control mice died. Although we did not investigate this further, it is possible that enough nonspecific immunity is induced by gene gun inoculation to shift the LD<sub>50</sub> curve.

The RSSE and CEE virus cross-reactive immunity that we observed was not surprising in that the prM and E polyprotein expression products of the two viruses are 94% identical. Nevertheless, it is known that certain E-specific monoclonal antibodies differentiate RSSE and CEE viruses and that minor changes in E can result in altered neuroinvasiveness in mice (17, 18). Consequently, although either of our DNA vaccines by itself may be sufficient for immunity to TBE-causing flaviviruses, it may be prudent to include both DNAs in a vaccine developed for humans.

In some of our experiments, not only did our candidate vaccines protect mice from death and illness after challenge, but they apparently prevented replication of the challenge virus, as indirectly measured by the absence of antibody titer increases and the absence of NS1-specific antibodies after challenge. Of course, neither of these methods is sensitive enough to detect low levels of virus replication, so it is possible that the challenge virus did establish an infection but was quickly eliminated. If sterile immunity did occur, we assume that it was related to neutralization of the challenge virus by circulating antibodies. Among the mechanistic possibilities for this are prevention of adsorption of virus to host cell receptors or alteration of the conformation of the viral envelope proteins to perturb entry of the virus into the host cell (7). Whichever mechanism occurred, sterile immunity was apparently not required for protective immunity. This is evidenced by the large increases in antibody titers after challenge of some of the mice in our duration of immunity experiments.

In conclusion, we feel that the DNA-gene gun technology offers great promise for a new generation of vaccines for TBE. For the future use of our vaccines, it is imperative that we demonstrate that they can elicit neutralizing and protective responses in primates as well as in mice. The technology is still new and is undergoing constant modifications and revisions. Nevertheless, gene gun inoculation of other DNAs, in quantities similar to those in our studies, effectively induced immune responses in larger animals such as pigs and nonhuman primates (9, 10). Thus, we expect that the amount of DNA needed for successful vaccination will not present a technical barrier. Also, because gene gun delivery of a candidate virus vaccine for hepatitis B (Geniva) was recently approved for use in a human clinical trial, we anticipate no regulatory obstacles for its eventual use in TBE vaccines for humans.

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#### REFERENCES

- Arikawa, J., A. L. Schmaljohn, J. M. Dalrymple, and C. S. Schmaljohn. 1989. Characterization of Hantaan virus envelope glycoprotein antigenic determinants defined by monoclonal antibodies. *J. Gen. Virol.* 70:615-624.
- Bos, J. D. 1997. The skin as an organ of immunity. *Clin. Exp. Immunol.* 107(Suppl. 1):3-5.
- Buckley, A. and E. A. Gould. 1985. Neutralization of yellow fever virus studied using monoclonal and polyclonal antibodies. *J. Gen. Virol.* 66:2523-2531.
- Calisher, C. H. 1988. Antigenic classification and taxonomy of flaviviruses (family Flaviviridae) emphasizing a universal system for the taxonomy of viruses causing tick-borne encephalitis. *Acta Virol.* 32:469-478. (Comments.)
- Calisher, C. H., N. Karabatsos, J. M. Dalrymple, R. E. Shope, J. S. Porterfield, E. G. Westaway, and W. E. Brandt. 1989. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. *J. Gen. Virol.* 70:37-43.
- Chu, Y.-K., C. Rossi, J. LeDuc, H. Lee, C. Schmaljohn, and J. Dalrymple. 1994. Serological relationships among viruses in the *Hantavirus* genus, family Bunyaviridae. *Virology* 198:196-204.
- Dimmock, N., J. 1995. Update on the neutralisation of animal viruses. *Med. Virol.* 5:165-179.
- Eisenbraun, M. D., D. H. Fuller, and J. R. Haynes. 1993. Examination of parameters affecting the elicitation of humoral immune responses by particle bombardment-mediated genetic immunization. *DNA Cell Biol.* 12:791-797.
- Fuller, D. H., M. Murphey-Corb, J. Clements, S. Barnett, and J. R. Haynes. 1996. Induction of immunodeficiency virus-specific immune responses in rhesus monkeys following gene gun-mediated DNA vaccination. *J. Med. Primatol.* 25:236-241.
- Fuller, J. T., D. H. Fuller, D. McCabe, J. R. Haynes, and G. Wiedera. 1995. Immune responses to hepatitis B virus surface and core antigens in mice, monkeys, and pigs after Accell particle-mediated DNA immunization. *Ann. N.Y. Acad. Sci.* 772:282-284.
- Fynan, E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson. 1993. DNA vaccines: protective immunizations by parental, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. USA* 90:11478-11482.
- Gould, E. A., A. Buckley, A. D. T. Barrett, and N. Cammack. 1986. Neutralizing (54K) and non-neutralizing (54K and 48K) monoclonal antibodies against structural and non-structural yellow fever virus proteins confer immunity in mice. *J. Gen. Virol.* 67:591-595.
- Haynes, J. R., D. H. Fuller, M. D. Eisenbraun, M. J. Ford, and T. M. Pertmer. 1994. Accell particle-mediated DNA immunization elicits humoral, cytotoxic, and protective immune responses. *AIDS Res. Hum. Retroviruses* 10(Suppl. 2):S43-S45.
- Heinz, F., and C. Kunz. 1977. Characterization of tick-borne encephalitis virus and immunogenicity of its surface components in mice. *Acta Virol.* 21:308-316.
- Heinz, F. X., S. L. Allison, K. Stiasny, J. Schlich, H. Holzmann, C. W. Mandl, and C. Kunz. 1995. Recombinant and virion-derived soluble and particulate immunogens for vaccination against tick-borne encephalitis. *Vaccine* 13:1636-1642.
- Heinz, F. X., R. Berger, W. Tuma, and C. Kunz. 1983. A topological and functional model of epitopes on the structural glycoprotein of tick-borne encephalitis virus defined by monoclonal antibodies. *Virology* 126:525-537.
- Holzmann, H., K. Stiasny, M. Ecker, C. Kunz, and F. X. Heinz. 1997. Characterization of monoclonal antibody-escape mutants of tick-borne encephalitis virus with reduced neuroinvasiveness in mice. *J. Gen. Virol.* 78:31-37.
- Holzmann, H., M. S. Vorobyova, I. P. Ladyzhenskaya, E. Ferenczi, M. Kundi, C. Kunz, and F. X. Heinz. 1992. Molecular epidemiology of tick-borne encephalitis virus: cross-protection between European and Far Eastern subtypes. *Vaccine* 10:345-392.
- Iacono-Connors, L., J. Smith, T. Ksiazek, C. Kelley, and C. Schmaljohn. 1996. Characterization of Langat virus antigenic determinants defined by monoclonal antibodies to E, NS1 and prM and identification of a protective, non-neutralizing prM-specific monoclonal antibody. *Virus Res.* 43:125-136.
- Kaufman, B. M., P. L. Summers, D. R. Dubois, and K. H. Eckels. 1987. Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. *Am. J. Trop. Med. Hyg.* 36:427-434.
- Kimura-Kuroda, J., and K. Yasui. 1988. Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies. *J. Immunol.* 141:3606-3610.
- Konishi, E., and P. W. Mason. 1993. Proper maturation of the Japanese encephalitis virus envelope glycoprotein requires cosynthesis with the pre-membrane protein. *J. Virol.* 67:1672-1675.
- Konishi, E., S. Pincus, E. Paoletti, W. W. Laegreid, R. E. Shope, and P. W. Mason. 1992. A highly attenuated host range-restricted vaccinia virus strain, NYVAC, encoding the prM, E, and NS1 genes of Japanese encephalitis virus prevents JEV viremia in swine. *Virology* 190:454-458.
- Konishi, E., S. Pincus, E. Paoletti, W. W. Laegreid, R. E. Shope, and P. W. Mason. 1992. Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. *Virology* 188:714-720.
- Kozak, M. 1989. The scanning model for translation: an update. *J. Cell. Biol.* 108:229-241.
- Kunz, C., F. X. Heinz, and H. Hofmann. 1980. Immunogenicity and reactivity of a highly purified vaccine against tick-borne encephalitis. *J. Med. Virol.* 6:103-109.
- Labuda, M., J. M. Austyn, E. Zuffova, O. Kozuch, N. Fuchsberger, J. Lysy, and P. A. Nuttall. 1996. Importance of localized skin infection in tick-borne encephalitis virus transmission. *Virology* 219:357-366.
- Lu, S., J. Arthos, D. C. Montefiori, Y. Yasutomi, K. Manson, F. Mustafa, E. Johnson, J. C. Santoro, J. Wissink, J. I. Mullins, J. R. Haynes, N. L. Letvin,

- M. Wyand, and H. L. Robinson. 1996. Simian immunodeficiency virus DNA vaccine trial in macaques. *J. Virol.* 70:3978-3991.
29. Mason, P. W., J. M. Dalrymple, M. K. Gentry, J. M. McCown, C. H. Hoke, D. S. Burke, M. J. Fournier, and T. L. Mason. 1989. Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. *J. Gen. Virol.* 70:2037-2049.
  30. Mason, P. W., S. Pincus, M. J. Fournier, T. L. Mason, R. E. Shope, and E. Paoletti. 1991. Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. *Virology* 180:294-305.
  31. Mathews, J. H., and J. T. Roehrig. 1984. Elucidation of the topography and determination of the protective epitopes on the E glycoprotein of Saint Louis encephalitis virus by passive transfer with monoclonal antibodies. *J. Immunol.* 132:1533-1537.
  32. Meegan, J. M., R. J. Yedloutschnig, B. A. Peleg, J. Shy, C. J. Peters, J. S. Walker, and R. E. Shope. 1987. Enzyme-linked immunosorbent assay for detection of antibodies to Rift Valley fever virus in ovine and bovine sera. *Am. J. Vet. Res.* 48:1138-1141.
  33. Monath, T. P., and F. X. Heinz. 1996. Flaviviruses, p. 961-1034. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Fields virology*, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
  - 33a. National Institutes of Health. 1996. Guide for the care and use of laboratory animals. National Institutes of Health, Bethesda, Md.
  34. Pertmer, T. M., M. D. Eisenbraun, D. McCabe, S. K. Prayaga, D. H. Fuller, and J. R. Haynes. 1995. Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA. *Vaccine* 13:1427-1430.
  35. Pincus, S., P. W. Mason, E. Konishi, B. A. Fonseca, R. E. Shope, C. M. Rice, and E. Paoletti. 1992. Recombinant vaccinia virus producing the prM and E proteins of yellow fever virus protects mice from lethal yellow fever encephalitis. *Virology* 187:290-297.
  36. Rambukkana, A., J. D. Bos, D. Irik, W. J. Menko, M. L. Kapsenberg, and P. K. Das. 1995. In situ behavior of human Langerhans cells in skin organ culture. *Lab. Invest.* 73:521-531.
  37. Rice, C. M. 1996. Flaviviridae: the viruses and their replication, p. 931-959. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), *Fields virology*, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
  38. Russell, P. K., W. E. Brandt, and J. M. Dalrymple. 1980. Chemical and antigenic structure of flaviviruses, p. 503-529. *In* R. W. Schlesinger (ed.), *The togaviruses*. Academic Press, New York, N.Y.
  39. Schalich, J., S. L. Allison, K. Stiasny, C. W. Mandl, C. Kunz, and F. X. Heinz. 1996. Recombinant subviral particles from tick-borne encephalitis virus are fusogenic and provide a model system for studying flavivirus envelope glycoprotein functions. *J. Virol.* 70:4549-4557.
  40. Stingl, G. 1993. The skin: initiation and target site of immune responses. *Recent Results Cancer Res.* 128:45-57.
  41. Ulmer, J. B., R. R. Deck, A. Yawman, A. Friedman, C. Dewitt, D. Martinez, D. L. Montgomery, J. J. Donnelly, and M. A. Liu. 1996. DNA vaccines for bacteria and viruses. *Adv. Exp. Med. Biol.* 397:49-53.
  42. Ulmer, J. B., J. J. Donnelly, R. R. Deck, C. M. DeWitt, and M. A. Liu. 1995. Immunization against viral proteins with naked DNA. *Ann. N.Y. Acad. Sci.* 772:117-125.
  43. Ulmer, J. B., J. C. Sadoff, and M. A. Liu. 1996. DNA vaccines. *Curr. Opin. Immunol.* 8:531-536.
  44. Whalen, R. G. 1996. DNA vaccines for emerging infectious diseases: what if? *Emerg. Infect. Dis.* 2:168-175.

# Sindbis Vectors Suppress Secretion of Subviral Particles of Japanese Encephalitis Virus from Mammalian Cells Infected with SIN-JEV Recombinants

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Double-subgenomic Sindbis virus (dsSIN) recombinants that express cassettes encoding prM-E or a C-terminally truncated form of E of Japanese encephalitis virus (JEV) were constructed. The products were efficiently expressed in both mammalian and mosquito cell lines infected with the dsSIN recombinants. However, suppression of prM-E secretion from mammalian cells infected with dsSIN-prM-E recombinants was observed. This suppression was more pronounced late in infection (<5% of total product was secreted during an 8-hr chase) than early in infection (15% secretion during a 6-hr chase). In comparison, a vaccinia virus-prM-E recombinant (vP829) described previously (E. Konishi *et al.* (1991) *Virology* 185, 401–410) was shown to secrete 35–50% of total product during a 6- to 8-hr chase both early and late in infection. In contrast, secretion of prM-E from dsSIN-prM-E-infected mosquito (C6/36) cells was found to be efficient (>50% during an 8-hr chase). The prM-E secreted from both mammalian and mosquito cells was in the form of subviral particles as determined by velocity gradient centrifugation, sensitivity to nonionic detergent, and analysis of processing of N-linked glycans. The truncated E protein expressed by the dsSIN recombinants was secreted efficiently from both mammalian and mosquito cells. Coinfection experiments with the dsSIN-JEV recombinants + wild-type vaccinia virus and vP829 + SIN demonstrated that the reduced level of secretion of subviral particles exhibited by the dsSIN-JEV recombinants was due to an inhibitory effect of the dsSIN vectors. Furthermore, this inhibitory effect was accounted for by the SIN nonstructural proteins since SIN replicons that express prM-E cassette in place of the SIN structural protein open reading frame exhibited a low level of subviral particle secretion. No self-propagating infectious particles were produced in cells transfected with SIN replicons that encode the JEV prM-E cassette. The suppression of subviral particle secretion was apparently correlated with the inhibition of cell protein synthesis which is mediated in SIN-infected vertebrate cells by expression of the SIN nonstructural proteins.

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## INTRODUCTION

Several virus-based vectoring systems for the expression of heterologous proteins are currently in use. Recently, a new expression system was developed based on two alphaviruses (members of the *Togaviridae* family): Sindbis virus (SIN) and Semliki Forest virus (SFV) (Bredenbeek and Rice, 1992; Liljeström and Garoff, 1991; Schlesinger, 1993; Xiong *et al.*, 1989; Hahn *et al.*, 1992). The alphavirus genome consists of a single-stranded RNA molecule of positive polarity that is roughly 11,800 bases in length (reviewed in Strauss and Strauss, 1994). The 5' two-thirds of the genomic RNA contains an open reading frame (ORF) which encodes replicase proteins necessary for the virus RNA synthesis. RNA amplification is initiated by the synthesis of a genome-length, negative-polarity RNA, which serves as a template for synthesis of both the genome RNA and a subgenomic RNA that consists of sequences from the 3' terminal one-third of the genome RNA and serves as an mRNA for the structural proteins. Synthesis of the subgenomic RNA is initiated at an internal promoter sequence (known as the subgenomic promoter) on the negative-polarity template.

Alphavirions consist of an icosahedral nucleocapsid composed of multiple copies of the capsid protein C and the viral RNA surrounded by a lipid bilayer of the cellular origin in which virus-encoded glycoproteins E1 and E2 are embedded.

Genome-length cDNA clones from which infectious RNA can be transcribed *in vitro* have been constructed for several alphaviruses (Rice *et al.*, 1987; Davis *et al.*, 1989; Liljeström *et al.*, 1991; Kuhn *et al.*, 1991). Two types of expression vectors based on the genomic cDNA clones of SIN and SFV have been developed, both of which take advantage of the high level of subgenomic RNA that accumulates in alphavirus-infected cells (reviewed by Bredenbeek and Rice, 1992). One type of vector, termed dsSIN, has two subgenomic promoters leading to synthesis of two subgenomic RNAs: one which directs synthesis of the heterologous protein and the second which directs synthesis of the viral structural proteins (Hahn *et al.*, 1992; Raju and Huang, 1991). This vector is self-replicating and capable of production of infectious viral particles. In the second type of vector, the heterologous sequences replace the structural protein ORF (Xiong *et al.*, 1989; Liljeström and Garoff, 1991). When introduced into cells, this type of vector self-replicates and hence is known as a replicon. Although repli-

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cons give rise to high levels of heterologous protein production, they are designed not to produce infectious particles unless the cells are cotransfected with defective helper RNAs that produce the structural proteins. Recently, however, cells transfected with an SFV replicon that contained the G glycoprotein of vesicular stomatitis virus (VSV) were found to release infectious particles that consisted of the replicon RNA surrounded by a lipid envelope in which the G protein was embedded (Rolls *et al.*, 1994).

Our long-term goal is to develop an expression and vaccine vector based on a live, attenuated vaccine strain of rubella virus. Rubella virus is a member of the *Rubivirus* genus of the *Togaviridae* family and has a replication strategy similar to the alphaviruses (reviewed in Frey, 1994). As a first step in this project, we examined the properties of dsSIN recombinants expressing proteins of Japanese encephalitis virus (JEV), one of the most important human pathogens of the family *Flaviviridae* (Shope, 1980; Monath, 1986). The flavivirus genome is a single-stranded, positive-polarity RNA of roughly 11,000 nucleotides in length. Flavivirions consist of a nucleocapsid containing the genomic RNA and multiple copies of a capsid protein C (14 kDa), which is surrounded by a lipid bilayer with two embedded virus-specified proteins: a small nonglycosylated membrane protein, M (7–8 kDa), and an envelope protein, E (55–60 kDa) (Heinz and Roehrig, 1990). The flavivirus genomic RNA contains a single long ORF which is translated into a large precursor that is proteolytically processed by cellular and virus-specific proteases. In the polyprotein precursor, these individual proteins are in the order C–prM–E–NS1–NS2A–NS2B–NS3–NS4A–NS4B–NS5, where prM is a glycosylated precursor of the M protein and NS1 through NS5 are nonstructural proteins (reviewed by Chambers *et al.*, 1990).

Previous studies using a vaccinia virus recombinant that expresses a JEV prM-E cassette (vP829) demonstrated efficient secretion from infected cells of subviral particles consisting of membrane containing both prM and E. vP829 induced protective immunity against neurovirulent JEV when used to inoculate mice (Konishi *et al.*, 1991, 1992). We constructed dsSIN recombinants that expressed similar prM-E cassettes. Interestingly, suppression of subviral particle secretion was observed in mammalian cells infected with dsSIN-prM-E recombinants. In this report, we describe our studies on this phenomenon, which show that suppression of subviral particle secretion is mediated by the SIN nonstructural proteins.

## MATERIALS AND METHODS

### Cell lines, plasmids, and virus strains

SIN plasmid vectors were provided by C. M. Rice (Washington University School of Medicine). The plasmids pTE5'2J and pTE3'2J (Hahn *et al.*, 1992; Raju and

Huang, 1991; Bredenbeek and Rice, 1992) both contain a cDNA copy of SIN genome and are designed for construction of SIN recombinants that express heterologous products from inserts situated either before (5' recombinants) or after (3' recombinants) the structural protein ORF in the SIN genome. The plasmid pSINrep5 (Bredenbeek *et al.*, 1993) is designed to generate recombinant replicons that express heterologous products from inserts cloned in place of the SIN structural proteins. The vaccinia virus recombinant vP829, which expresses a JEV prM-E cassette, has been previously described (Konishi *et al.*, 1991, 1992). Vaccinia virus recombinants vNS1234 (which expresses the SIN nonstructural proteins), vNS1 × 2 × 3 × 4(s) (in which the cleavage sites between the individual nonstructural proteins have been mutated leading to production of precursor for the SIN nonstructural proteins; Lemm and Rice, 1993), and vTM3/SIN/S (which expresses the SIN structural proteins; B. Prágai and C. M. Rice, unpublished) were provided by C. M. Rice. The vaccinia virus recombinant vTF7-3, which expresses T7 RNA polymerase (Fuerst *et al.*, 1986), was obtained from B. Moss. The LHD-J strain of vaccinia virus (Stern and Dales, 1974) was used as a wild-type vaccinia virus strain. Unless otherwise specified, BHK-21 cells (called BHK cells throughout the text of the article), Vero cells, and HeLa cells were maintained at 35° in Dulbecco's MEM (DMEM) supplemented with 10% fetal bovine serum (FBS). The C6/36 mosquito cell line (*Aedes Albopictus*) was grown at 28° as described by Tesh (1979). All experiments with mammalian cells were done at 35° and all experiments with C6/36 cells were done at 28°.

### Construction of recombinant plasmids

Restriction enzymes, calf intestinal alkaline phosphatase, and T4 DNA ligase were obtained from New England Biolabs or Boehringer Mannheim Biochemicals. Standard recombinant DNA techniques were used (Maniatis *et al.*, 1982) with minor modifications for cloning, screening, and plasmid purification. Nucleic acid sequences were confirmed using a dideoxy chain-termination sequencing kit (United States Biochemicals, Cleveland, OH).

The plasmid pARJ1a (P. W. Mason, unpublished data), which was constructed by combining two JEV cDNA clones, PM-7 and PM-6 (McAda *et al.*, 1987), and contains the C-prM-E-NS1 encoding region of JEV genome, was used as a template for generation of the JEV cassettes which were introduced into the SIN vectors: 80%E fragment and two variants of prM-E cassette (with different length of prM signal sequence; Fig. 1B). These cassettes were synthesized by means of PCR using thermostable Deep Vent DNA polymerase (New England Biolabs) and sets of synthetic oligonucleotide primers: 5'-GCTCTAGACCATGAACAAAAGAGGAGG and 5'-GCACATCTAGATCAAGCATGCACATTGG for the v5'ME, v3'ME, and repME constructs; 5'-GCGTCTAGACCATGT-

GGCTCGCGAGCTTG and 5'-GCACATCTAGATCAAGCA-TGCACATTGG for vM3'ME and MrepME; and 5'-CGA-TACTCTAGACCATGCTTGGCAGTAAC and 5'-GCCTCT-AGATCATCCAGCCTTGTGCC for v5'80%E and v3'80%E. In each pair, both primers contained an *Xba*I site (underlined) to facilitate cloning into the *Xba*I site in the poly-linker region in the SIN vectors. Since all cassettes were from internal sequence of the JEV ORF, artificial start and stop codons were included into each pair of primers (bold underlined). In the PCR reactions 20 ng of pARJ1a was combined with 0.5  $\mu$ g of each primer in a 100- $\mu$ l vol of Vent DNA polymerase buffer (New England Biolabs) containing 200  $\mu$ M dNTPs and 20 units/ml Deep Vent DNA polymerase. A protocol of 25 cycles of 1 min @ 92°, 2 min @ 45°, and 2 min @ 72° was employed. The PCR products were digested with *Xba*I and ligated with dephosphorylated *Xba*I-linearized pTE5'2], pTE3'2], or pSINrep5. Competent MC1061 *Escherichia coli* cells were then transformed with the ligation mixtures and the plasmids containing the JEV insert in the desired orientation were identified by restriction analysis.

#### Preparation of the dsSIN recombinant viruses

Recombinant SIN expression plasmids containing JEV cassettes were digested with *Xho*I, phenol-chloroform extracted, and ethanol precipitated. The linearized templates (roughly 0.5  $\mu$ g per transcription) were transcribed *in vitro* using 25 units of SP6 RNA polymerase (Epicentre Technologies) for 1 hr at 37° in a 25- $\mu$ l volume of buffer provided by the manufacturer containing 1 mM NTPs (Pharmacia), 2 mM 5' cap analog (7<sup>m</sup>G5'ppp5'G), and 40 units of RNasin (New England Biolabs). Approximately 1  $\mu$ g of each RNA transcript was used for electroporation of BHK cells according to the procedure of Liljeström *et al.* (1991). Media was collected from cells electroporated with infectious constructs 20–24 hr after plating, clarified, and stored at –70°. Cells electroporated with SIN-JEV replicons were radiolabeled for analysis 16 hr after plating.

#### Infection of the cells and radiolabeling

BHK, Vero, HeLa, or C6/36 cell monolayers grown in 24-mm plates were infected with SIN or dsSIN-JEV recombinants diluted in maintenance medium at an m.o.i. of 10 PFU/cell. BHK cells were infected with vaccinia virus recombinants at an m.o.i. of 10 PFU/cell. In coinfection experiments, BHK cell monolayers were infected at an m.o.i. of 10 PFU/cell for each virus. Following adsorption for 1 hr, the virus inoculum was removed and the cells were incubated in DMEM containing 2% FBS. At the time postinfection indicated in each experiment, the medium was replaced with methionine (Met)-free medium containing 2% FBS. 45 min later [<sup>35</sup>S]methionine (Amersham; 1000 Ci/mmol) was added to a concentration of 100  $\mu$ Ci/ml. Following incubation for 1 hr the medium containing radiolabel was removed, the cells were rinsed

two times with PBS, maintenance medium containing no radiolabel was added, and the cells were incubated for the indicated time and then lysed as described below. In cycloheximide treatment experiments, radiolabeling was for 20 min followed by a chase with maintenance medium containing 50  $\mu$ g/ml of cycloheximide.

#### Radioimmunoprecipitations and polyacrylamide gel electrophoresis

Following the radiolabeling protocol, the culture fluid was removed from the cells, clarified by centrifugation at 7000 *g* for 5 min, and frozen at –70° until use. The cell monolayer was rinsed with PBS, lysed in a volume of RIP buffer [1% NP-40, 0.5% Na deoxycholate, 0.1% SDS in TNE buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 3 mM EDTA)] equal to the volume of the removed culture fluid, clarified by centrifugation, and frozen at –70° until use. Two monoclonal antibodies (MAbs), one specific for the JEV E protein (J3-11B9; Mason, 1989) and the other specific for the JEV prM/M protein (J2-2F1; Mason *et al.*, 1987) were used for radioimmunoprecipitation (RIP). Equal aliquots of the culture fluids and cell lysates (usually 100  $\mu$ l) were used in RIPs. Prior to RIP, one-tenth volume of 10X-concentrated mixture of RIP detergents (10% NP-40, 5% Na deoxycholate, 1% SDS in TNE buffer) was added to the culture fluids. Each 100- $\mu$ l aliquot was mixed with 1  $\mu$ l of ascitic fluid containing the MAb and incubated overnight at 4°. One-eighth volume of a 50% suspension of protein A-Sepharose (Pharmacia) prepared in RIP wash buffer (0.5% NP-40, 0.5% Na deoxycholate in TNE) was then added to each RIP sample and the samples were rotated for 1 hr at room temperature. The protein A-Sepharose was pelleted by microcentrifugation, washed three times with RIP wash buffer, and suspended in 20  $\mu$ l of sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5%  $\beta$ -mercaptoethanol, 10% glycerol, 0.02% bromophenol blue). The bound JEV antigens were released from the protein A-Sepharose by boiling for 2 min. Following pelleting of the protein A-Sepharose beads, the proteins in the supernatant were resolved on 7.5–25% gradient polyacrylamide minigels (Hoefer) (Laemmli, 1970). Following electrophoresis the gels were fixed for 15 min in 10% ethanol, 7% acetic acid, dried, and autoradiographed. The dried gels were also exposed to a Fuji imaging plate for 1–3 hr and subsequently the plate was scanned in a Fujix BAS1000 Bio Imaging Analyzer (Fuji Photo Film, Japan). The intensity of radiation corresponding to each individual protein band was measured using software provided by the manufacturer. The efficiency of protein secretion as expressed throughout the text was calculated as the percentage of labeled secreted protein out of total amount of radiolabeled protein (secreted plus intracellular).

#### Velocity gradient centrifugation

Two hundred microliters of clarified culture fluid from radiolabeled cells was applied to a 5–20% continuous

sucrose gradient [w/w, prepared with buffer A (10 mM Tris-HCl, pH 7.5, 0.4 M NaCl, 1 mM EDTA)]. A second sample, to which 0.1% Triton X-100 was added, was applied to a parallel 5–20% sucrose gradient prepared with buffer A containing 0.1% Triton X-100. The gradients were centrifuged for 3.5 hr at 35,000 rpm in SW.41 rotor, Beckman L8-M ultracentrifuge, at 4°. Aliquots (50  $\mu$ l) of the fractions collected after centrifugation were combined with 1 ml of Opti-Fluor scintillation fluid (Packard) and counted by liquid scintillation spectroscopy. Aliquots (200  $\mu$ l) of the gradient fractions were also diluted with an equal volume of 2X RIP detergents (2% NP-40, 1% Na deoxycholate, 0.2% SDS) and the proteins were immunoprecipitated and analyzed as described above.

#### Indirect immunofluorescence assay (IFA)

BHK cell monolayers grown in eight-well chamber slides for IFA (Nunc) were infected or coinfecting with indicated viruses at an m.o.i. of 10 PFU/cell for each virus. Cells electroporated with SIN replicon transcripts were seeded into similar slides. At the indicated times postinfection/post-transfection, cells were washed three times with PBS (10 mM Na phosphate, pH 7.5, 0.14 M NaCl; these washes were repeated after each of the following incubations) and fixed with 95% ethanol for 10 min. For double-staining, monolayers were incubated for 30 min with a mixture of MAb J3-11B9 (specific for the JEV E protein; Mason *et al.*, 1987) and a rabbit polyclonal antisera against SIN virions, both diluted 1:100 in PBS containing 1% BSA. For single-staining, cells were incubated with MAb J3-11B9. The monolayers were then incubated with a mixture of FITC-conjugated goat anti-rabbit IgG (Gibco-BRL, diluted 1:100) and biotinylated goat anti-mouse IgG (Gibco-BRL, diluted 1:100) (or biotinylated goat anti-mouse IgG alone). Finally, the monolayers were incubated with streptavidin-Texas Red conjugate (Gibco-BRL). The slides were examined using a Zeiss Axioplan microscope with epifluorescence capacity.

## RESULTS

#### Expression of JEV proteins by dsSIN vectors

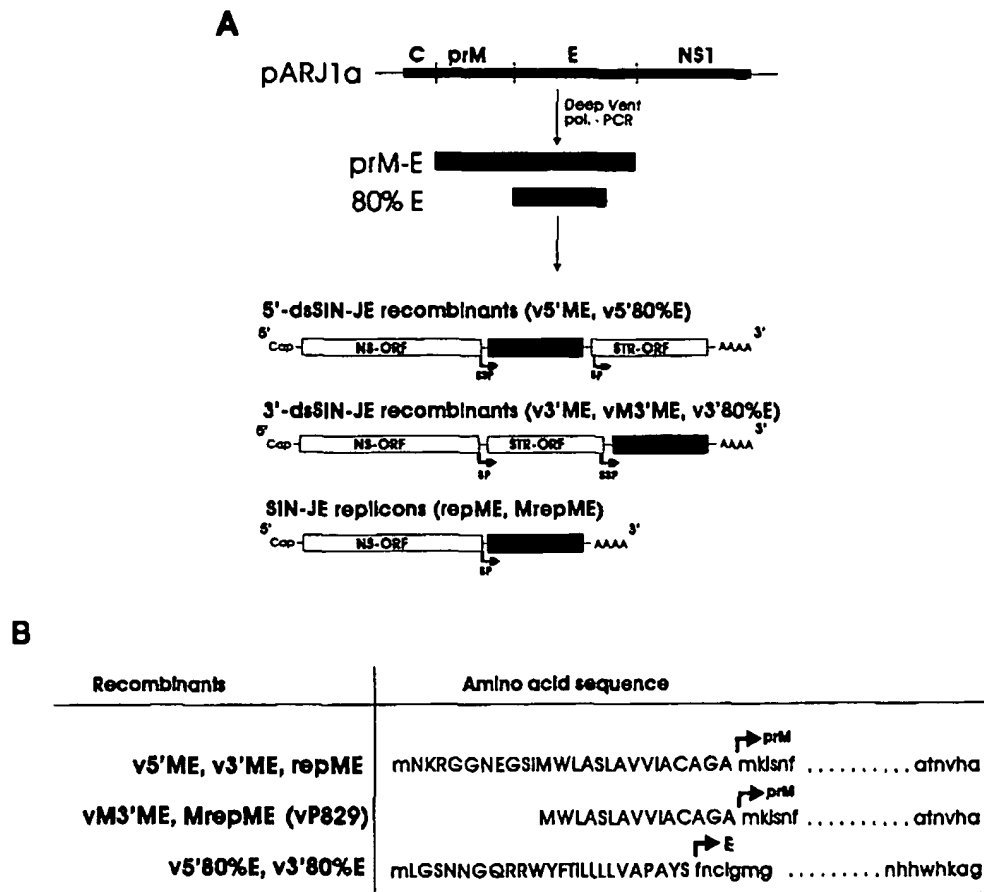
dsSIN recombinants containing the prM-E cassette in both the 5' (v5'ME) and 3' (v3'ME, vM3'ME) cloning sites were constructed (depicted schematically in Fig. 1A). Two versions of the prM-E cassette were introduced into dsSIN vectors. Both versions ended at the carboxy terminus of E, but they differed in the number of codons upstream from the amino terminus of prM which they contained (Fig. 1B). Of these, the version in the vM3'ME construct is identical to the cassette in vP829, a vaccinia virus-prM-E recombinant (Konishi *et al.*, 1991). Since these cassettes were from the interior of the JEV ORF, artificial initiation and termination codons were placed at the 5' and 3' ends of the cassettes.

Initially, a protocol for analysis of expression com-

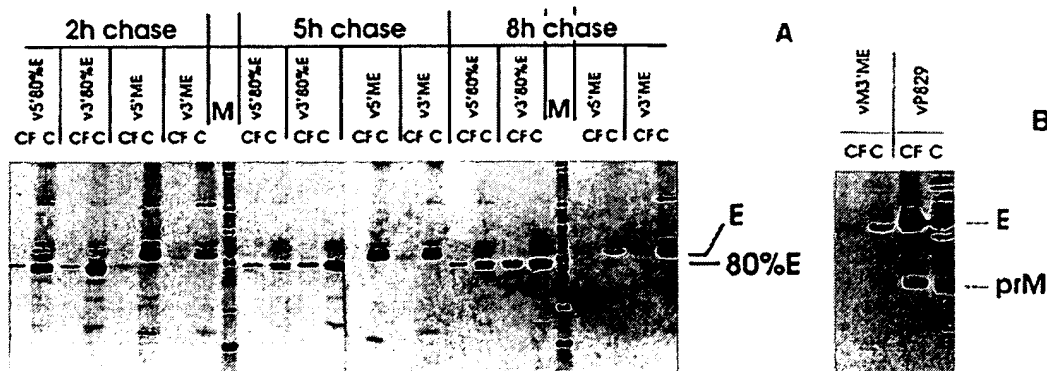
monly used for vaccinia virus (VV) recombinants (1-hr pulse-labeling at 14–16 hr postinfection followed by chases up to 8 hr) was employed. In infected BHK cells, the v5'ME, v3'ME, and vM3'ME recombinants all produced proteins that comigrated with prM and E expressed by vP829 and were recognized by MAbs specific for prM and E (expression of E by the dsSIN vectors is shown in Figs. 2A and 2B; data for prM is not shown). Unlike previous reports that indicate that expression of foreign proteins was higher in 3' vector (Bredenbeek and Rice, 1992), expression of E appeared similar in both 3' and 5' vectors. Interestingly, only 5% of the E protein synthesized accumulated in the extracellular medium, even after an 8-hr chase. Inefficient secretion was also observed in Vero and HeLa cells infected with the dsSIN-prM-E recombinants (data not shown). It was shown previously with vP829 that a large percentage of prM and E proteins synthesized in infected HeLa cells was released into the culture fluid in the form of empty viral envelopes, with a  $t_{1/2}$  for secretion of 6 hr (Konishi *et al.*, 1991). As shown in Fig. 2B, vP829 efficiently secreted E protein from infected BHK cells; 60% of the total product was present in the culture fluid following an 8-hr chase.

VV recombinants that express the complete dengue virus E protein do not release the protein from cells due to intracellular targeting of the product (Bray *et al.*, 1989; Men *et al.*, 1991). However, E protein constructs that contain the N-terminal 80% of the protein (and thus lack the transmembrane anchor which is at the COOH end of the protein) are efficiently secreted (Men *et al.*, 1991). Accordingly, dsSIN-JEV recombinants that express truncated E cassette which contains the E protein signal sequence and the N-terminal 80% of the protein (termed 80%E) were constructed (shown schematically in Fig. 1). The truncated product was found to be efficiently secreted from BHK cells infected with these recombinants (Fig. 2A; ~40% of the total radiolabeled product was present in the culture medium after an 8-hr chase). Thus, the secretion pathway per se is not affected in dsSIN recombinant-infected cells for an individual polypeptide. The reduced efficiency of prM-E secretion in comparison with 80%E could not be attributed to a higher toxicity of dsSIN-prM-E recombinants, since coinfection of BHK cells with v3'ME and v3'80%E did not affect secretion of truncated E (data not shown). Interestingly, the intracellular 80%E product migrated electrophoretically as a double band. The more rapidly migrating band diminished with the increased chase periods and the less rapidly migrating band was the major product detected in the culture medium.

Alphavirus infection of mammalian culture cells is accompanied by inhibition of host macromolecular synthesis and profound cytopathic effect (CPE) (Wengler, 1980). The experiments described thus far were done relatively late in the SIN replication cycle when CPE was observed. Development of CPE is delayed in dsSIN-prM-E-infected



**FIG. 1.** SIN-JEV recombinant viruses used in the study. (A) pARJ1a, a plasmid which contains a cDNA copy of the C-prM-E-NS1 region of JEV genome, was used to obtain prM-E and 80%E cassettes by means of PCR using the sets of primers given under Materials and Methods. The PCR fragments were cloned into *Xba*I site of pTE5'2J, pTE3'2J, or pSINrep vectors. The genome of the resulting recombinants is diagrammed. NS-ORF is the SIN nonstructural protein ORF, STR-ORF is the SIN structural protein ORF, the black box is the JEV cassette, and SP and SSP are subgenomic and second subgenomic promoters, respectively, from which SG RNAs are synthesized. In B, the amino- and carboxy-terminal sequences encoded by the cassettes are given. The beginning of prM and E according to McCada *et al.* (1987) is indicated by arrows, the sequence upstream serves as a signal sequence. The carboxy terminus of prM-E protein constructs is the authentic COOH-terminus of the E protein while the COOH-terminus of the 80%E is at residue 400 of the E protein (out of 500).



**FIG. 2.** Expression of E and 80%E by the dsSIN recombinants and vP829. (A) BHK cells infected with the dsSIN recombinants v5'ME, v3'ME, v5'80%E, or v3'80%E were labeled at 16 hr postinfection for 1 hr with [ $^{35}$ S]Met and then chased with cold medium for 2, 5, or 8 hr. (B) BHK cells infected with vM3'ME or vP829 were radiolabeled at 16 hr postinfection for 1 hr and chased for 8 hr. Equal fractions of the cell lysate (C) and culture fluid (CF) were immunoprecipitated with a Mab specific for E and then subjected to SDS-PAGE analysis. The positions of migration of the E and 80%E products are denoted in the right margin. "M" lanes contain molecular weight markers (200, 97, 68, 45, 29, 18.4, and 14.3 kDa, respectively). In vP829-infected cells, a visible amount of intra- and extracellular prM protein was detected which was coprecipitated with E.

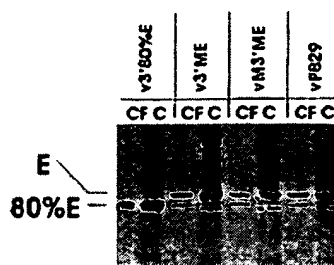


FIG. 3. Secretion of JEV-specific products expressed by dsSIN recombinants and vP829 early in infection. BHK cells infected with v3' 80%E, v3'ME, vM3'ME, or vP829 were labeled at 5 hr postinfection with [ $^{35}$ S]-Met for 1 hr and then chased for 6 hr. Equal fractions of the cell lysates (C) and culture fluids (CF) were immunoprecipitated with an anti-E MAb and analyzed by SDS-PAGE. The positions of migration of the E and 80%E products are denoted.

cells in comparison to dsSIN-infected cells; in our experience dsSIN recombinants that contain large cassettes replicate more slowly, thus causing the delay in CPE (J.-P. Chen and K. V. Pugachev, unpublished observations). An experiment was done using infected BHK cells early in the replication cycle when no CPE was observable. Cells infected with the recombinant viruses were radiolabeled at 5 hr postinfection for 1 hr and chased for 6 hr. As shown in Fig. 3, secretion of E in cells infected with vP829 was more efficient (35%) than in cells infected with either dsSIN-prM-E recombinant (16 and 15% for v3'ME and vM3'ME, respectively), although the disparity was not as pronounced as when the experiment was done late in the replication cycle.

In contrast to mammalian cell lines, SIN replication in mosquito cell lines is not accompanied by inhibition of cell macromolecular synthesis or CPE. Therefore, expression of prM-E in mosquito C6/36 cells infected with dsSIN-JEV recombinants was analyzed. As shown in Fig. 4, in cells labeled for 1 hr at 16 hr postinfection followed by an 8-hr chase, up to 60% of the labeled E protein was present in the medium. In cells infected with v5'80%E and v3'80%E, 40% of the labeled 80%E was present in the culture fluid. Interestingly, the 80%E product expressed in C6/36 cells migrated as a single band, unlike the double-banded product from BHK cells.

#### The JEV proteins secreted from dsSIN-prM-E recombinant-infected cells are in the form of subviral particles

The extracellular prM and E glycoproteins produced by vP829 are in the form of subviral particles and it has been shown that production of subviral particles is an important factor in the ability of vP829 to induce a protective immune response (Konishi *et al.*, 1991, 1992). Sucrose density gradient centrifugation was undertaken to define the physical form of the JEV-specific products in the extracellular fluids harvested from BHK cells infected with v5'ME or vP829 and C6/36 cells infected with v5'ME. The culture fluid from v5'80%E-infected BHK cells was

included as a control since no particulate products were expected to be formed by the 80%E protein. A peak of radioactivity was detected in gradients of media from vP829- and v5'ME-infected BHK and v5'ME-infected C6/36 cells while no peak was present in gradients of v5'80%E-infected BHK cells (Fig. 5A). As expected, the amount of material in the peak from the culture fluid from v5'ME-infected C6/36 cells and vP829-infected BHK cells was several-fold higher than in the corresponding peak from culture fluid of v5'ME-infected BHK cells. The peak from the culture fluid from vP829-infected cells was previously shown to consist of subviral particles (Konishi *et al.*, 1991, 1992). As expected, these peaks contained both prM and E as detected by RIP (data not shown) and treatment of the culture medium with Triton X-100 prior to centrifugation eliminated the peaks (Fig. 5B). The peak from v5'ME-infected BHK cells comigrated with the subviral particle peak produced by vP829. However, the peak from v5'ME-infected C6/36 cells migrated one fraction slower than the vP829 peak. Whether this was due to variations between gradients or a difference in physical characteristics of the subviral particles derived from vertebrate and arthropod cells was not resolved. Endoglycosidase treatment analysis of oligosaccharide groups present on the extracellular and intracellular prM and E produced in both BHK and C6/36 cells infected with dsSIN recombinants showed that the secreted proteins had been converted from high-mannose to complex form (data not shown).

#### The inhibitory effect on secretion of the JEV subviral particles is mediated by the SIN nonstructural proteins

Coinfection experiments were undertaken to determine if the difference in efficiency of secretion of JEV subviral particles encoded by dsSIN or VV vectors was due to a positive effect exerted by the VV vector or a negative effect exerted by the dsSIN vectors. BHK cells

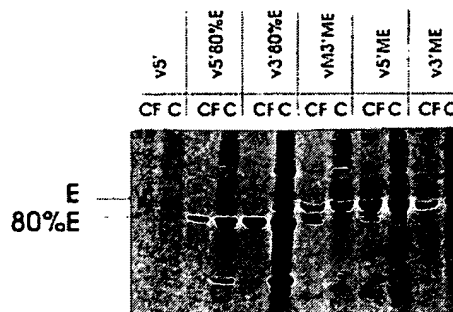


FIG. 4. Secretion of JEV-specific products from mosquito C6/36 cells infected with dsSIN recombinants. C6/36 cells infected with v5', v5'80%E, v3'80%E, vM3'ME, v5'ME, or v3'ME were labeled at 16 hr postinfection with [ $^{35}$ S]-Met for 1 hr and chased for 8 hr. Equal fractions of the cell lysates (C) and culture fluids (CF) were immunoprecipitated with an anti-E MAb and analyzed by SDS-PAGE. The positions of migration of the E and 80%E products are denoted.

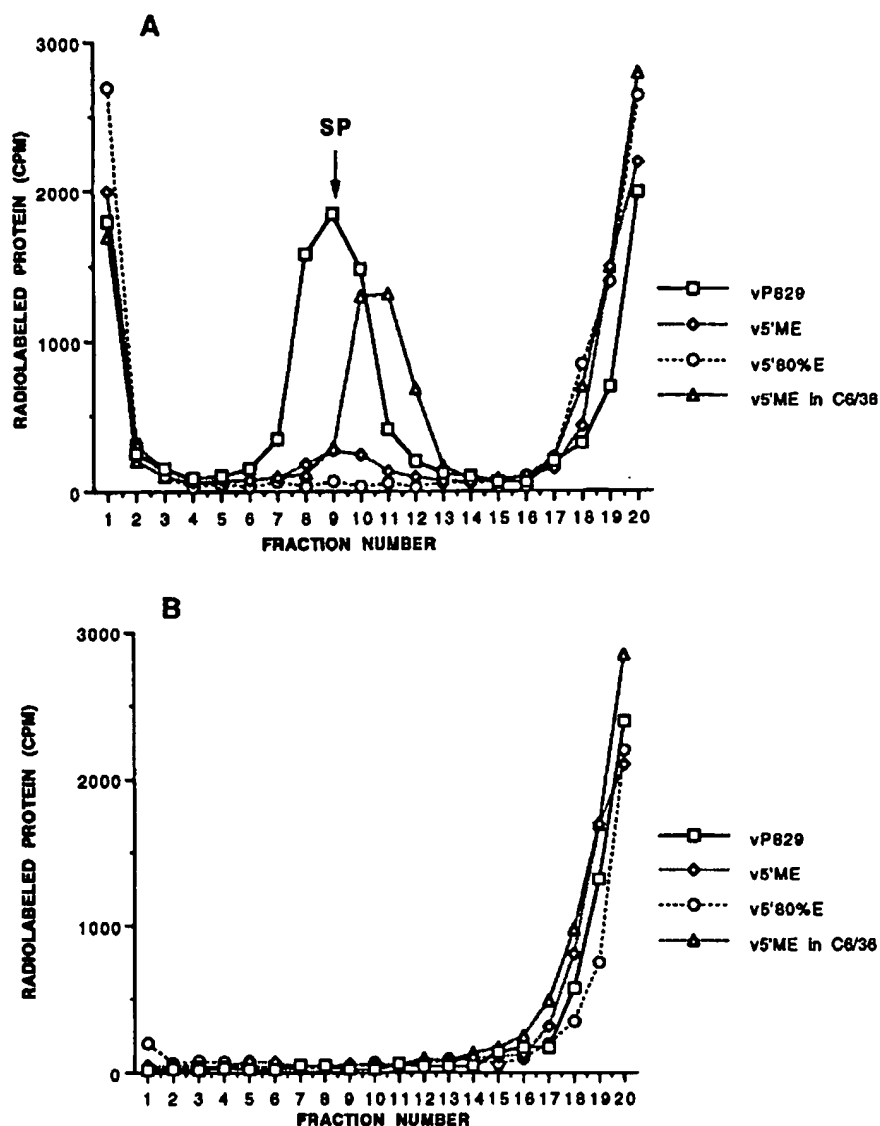


FIG. 5. Velocity centrifugation of prME complexes secreted from cells infected with recombinant viruses. Aliquots (200  $\mu$ l) of culture fluids harvested from BHK cells infected with vP829, v5'ME, or v5'80%E and C6/36 cells infected with v5'ME that were radiolabeled for 1 hr at 16 hr postinfection and chased for 8 hr were applied to 5–20% (w/w) continuous sucrose gradients (A). Duplicate 200- $\mu$ l aliquots were made 0.1% with Triton X-100 and applied to gradients containing 0.1% Triton X-100 (B). Gradients were centrifuged for 3.5 hr at 35,000 rpm. Fractions were collected and aliquots of the fractions were counted by liquid scintillation spectroscopy. The peak of JEV subviral particles (SP), which have been characterized previously for vP829 (Konishi *et al.*, 1991, 1992), is denoted by an arrow.

infected singly with wild-type VV (wtVV), vP829, the v5' SIN vector, or v5'ME or coinfecting with vP829 + v5' SIN or v5'ME + wtVV were radiolabeled at 16 hr postinfection for 1 hr followed by an 8-hr chase. A gel of whole cell lysates is shown in Fig. 6A. While coinfection did not induce a detectable change in the pattern of proteins produced by either virus, the amount of VV proteins produced in coinfecting cells was substantially (~fivefold) reduced while the amount of SIN proteins produced was unaffected. When the production and secretion of JEV E protein was analyzed by immunoprecipitation (Fig. 6B), it was found that wtVV exerted no effect on E expression by v5'ME. In contrast, coinfection resulted in a fourfold

decrease in the total amount of E produced by vP829, a decrease most likely reflective of the overall decrease of VV-specific proteins in coinfecting cells. Additionally, a suppression of secretion of E was observed; 20% of total E was in the cell medium in coinfecting cells in comparison with 60% in cells infected with vP829 alone. Thus, SIN infection appears to exert an inhibitory effect on secretion of JEV subviral particles.

It was demonstrated previously that SIN can replicate in cells infected with VV or VV recombinants (Rice *et al.*, 1985; Li *et al.*, 1991). However, the effect of SIN on VV replication was not analyzed and the results of coinfection experiments could be due to SIN preventing VV repli-

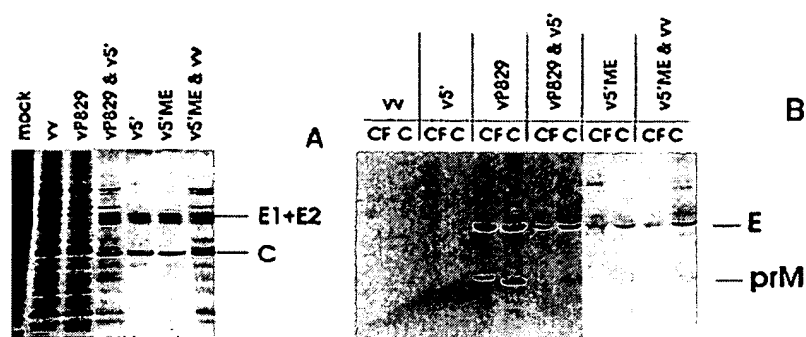


FIG. 6. Secretion of JEV-specific products from BHK cells coinfecting with vP829 + SIN and v5'ME + vaccinia virus. BHK cells mock-infected, infected with wild-type vaccinia virus (vv), SIN (v5'), vP829, or v5'ME or coinfecting with vP829 + SIN (vP829 and v5') or v5'ME + vv were labeled with [ $^{35}$ S]Met for 1 hr at 16 hr postinfection and chased for 8 hr. In A, an autoradiograph of a polyacrylamide gel of equal aliquots of whole cell lysates is shown. The position of migration of the SIN C and E1 + E2 is shown in the right margin. Equal fractions of the cell lysates (C) or culture fluids (CF) were also immunoprecipitated with an anti-E MAb and analyzed electrophoretically (B). In several cases, a visible amount of the coprecipitated prM protein was detected along with E.

cation in the majority of the coinfecting cells. To determine if both VV and SIN replicate in coinfecting cells, BHK cell monolayers were infected with SIN or vP829 alone or coinfecting with both viruses, and 14 hr postinfection, cells were analyzed by indirect IFA by double-staining with a MAb specific for the JEV E protein and polyclonal serum specific for the SIN structural proteins. Cells infected with SIN or coinfecting with SIN and vP829 were 100% positive for SIN antigens; similarly, 100% of cells infected with vP829 or coinfecting with vP829 and SIN were positive for the JEV E protein (data not shown). Thus, cells were productively coinfecting by both SIN and the VV recombinant.

To determine if the low level of secretion of JEV subviral particles from dsSIN-prM-E-infected mammalian cells was due to the presence of large amounts of SIN structural proteins in infected cells, recombinant SIN replicons containing the two variants of the JEV prM-E cassette were constructed (repME and MrepME; see Figs. 1A and 1B). When *in vitro* transcripts from these replicons were electroporated into BHK cells, no relative increase in secretion of JEV prM and E was observed in comparison with cells transfected with vM3'ME RNA (Fig. 7). Thus, the suppression of secretion appeared to be due to expression of the SIN nonstructural proteins by the replicon.

To determine if SIN replicons that express the JEV prM-E cassette are capable of production of self-propagating infectious particles (as demonstrated in cells transfected with recombinant SFV replicons that encoded the G protein of VSV; Rolls *et al.*, 1994), BHK cell monolayers were mock-transfected or transfected with SINrep or repME RNAs. 10, 24, and 36 hr post-transfection, cells were analyzed by indirect IFA using an anti-E MAb. Approximately 10% of cells transfected with repME were positive at 10 hr postinfection and this percentage remained the same at 24 and 36 hr postinfection (data not shown). When medium harvested from the monolayers transfected with repME at 24 hr post-transfection was added

to fresh BHK cells, no positive cells were subsequently detected by IFA (data not shown). Thus, SIN replicons encoding the JEV prM-E do not produce self-packaging infectious particles.

In an alternate approach to study the effect of the SIN nonstructural proteins on secretion of JEV prM and E, BHK cells were coinfecting with vP829 and one of three VV recombinants that each express one of the SIN ORFs: vNS1234, which expresses the nonstructural ORF; vNS1  $\times$  2  $\times$  3  $\times$  4(s), which expresses a mutagenized version of the nonstructural ORF in which all of the cleavage sites are blocked (Lemm and Rice, 1993); and vTM3/SIN/S, which expresses the structural ORF (B. Prágai and C. M. Rice, personal communication). In all of these recombinants, expression is dependent on T7 RNA polymerase which is provided by coinfection with VV recom-

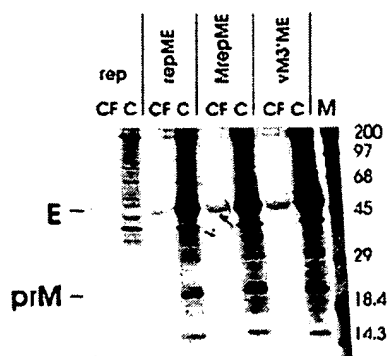


FIG. 7. Secretion of JEV E protein produced by SIN-prM-E replicons. BHK cells were electroporated with RNAs synthesized *in vitro* from pSINrep5, pRepME, pMRepME, and pM3'ME plasmid templates. Sixteen hours post-transfection, cells were labeled for 1 hr with [ $^{35}$ S]Met and chased for 8 hr. Equal fractions of the cell lysates (C) or culture fluid (CF) were immunoprecipitated with an MAb specific for E and then analyzed by SDS-PAGE. A visible amount of intracellular prM was coprecipitated with the MAb from several of the whole cell lysates. The "M" lane contains molecular weight standards whose MW (in kDa) is given in the right margin.

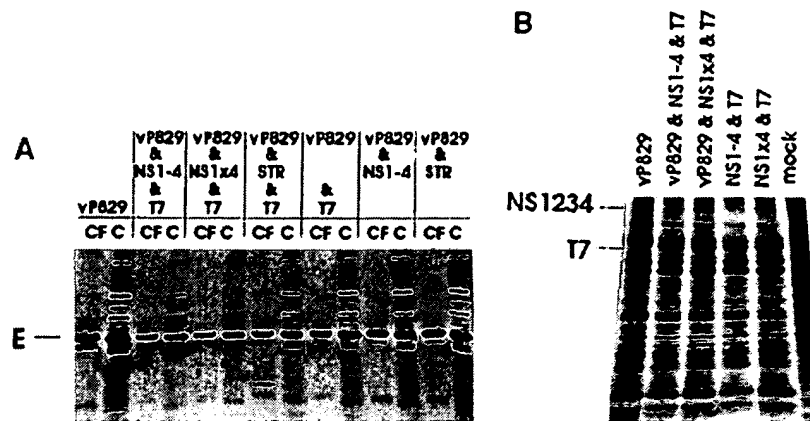


FIG. 8. Secretion of JEV E protein produced by vP829 in cells coinfecting with vaccinia recombinants that express SIN proteins. BHK cells infected with vP829 or coinfecting with vP829 + vNS1234 + vTF7-3, vP829 + vNS1  $\times$  2  $\times$  3  $\times$  4(s) + vTF7-3, vP829 + vTM3/SIN/S + vTF7-3, vP829 + vNS1234, or vP829 + vTM3/SIN/S were radiolabeled for 1 hr at 16 hr postinfection and chased for 8 hr. (A) Equal fractions of the cell lysates (C) or culture fluids (CF) were immunoprecipitated with an anti-E MAb and analyzed by SDS-PAGE. The position of migration of E is denoted. (B) Whole cell lysates were analyzed by SDS-PAGE. The positions of migration of T7 RNA polymerase and the uncleaved SIN nonstructural precursor (NS1234) are denoted. In the lane labels, vNS1234 is designated as NS1-4, vNS1  $\times$  2  $\times$  3  $\times$  4(s) as NS1  $\times$  4, vTM3/SIN/S as STR and vTF7-3 as T7.

binant vTF7-3. As shown in Fig. 8A, in cells coinfecting with vP829 and vTF7-3, vNS1234, or vTM3/SIN/S there was a two- to threefold depression in the total amount of E synthesized in comparison with cells infected singly with vP829. In triply infected cells (vP829 + vTF7-3 + vNS1234, vNS1  $\times$  2  $\times$  3  $\times$  4(s), or vTM3/SIN/S), only one-fourth to one-sixth as much E was synthesized as in vP829-infected cells. This effect was not specific to expression of the nonstructural ORF and is probably related to the presence of a number of different viruses in the same cells, reducing the relative amount of a product made by only one of the viruses. As can be seen in Fig. 8B, no alteration or inhibition of total VV-specific protein synthesis was detected in cells coinfecting with vP829 and vNS1234 or vNS1  $\times$  2  $\times$  3  $\times$  4(s), either in the presence or absence of vTF7-3. The secretion efficiency of E synthesized in vP829-infected cells (43%) was similar to cells coinfecting with vTF7-3 and vNS1234, vNS1  $\times$  2  $\times$  3  $\times$  4(s), or vTM3/SIN/S (36–43%).

#### Cycloheximide treatment experiment

The infection of cultured mosquito cells with alphaviruses is not cytopathic, whereas in mammalian cell cultures a profound CPE is produced (Brown and Condreay, 1986). The most impressive feature of the alphavirus-induced CPE in cultured vertebrate cells is an arrest of host-cell protein and RNA synthesis (Wengler, 1980). To determine if the inhibition of subviral particle secretion in dsSIN-infected cells was due to a shut-off of the cellular protein synthesis, v5'ME-infected C6/36 cells and vP829-infected BHK cells (in both of which normally high amounts of the extracellular subviral particles are produced) were radiolabeled for 20 min and then chased for 8 hr in the presence or absence of 50  $\mu$ g/ml cyclohexi-

imide. Figure 9 demonstrates that cycloheximide treatment did not noticeably affect secretion of either the subviral particles or 80%E.

#### DISCUSSION

In this study, a series of dsSIN recombinants that express prM-E and a C-terminal truncated form of E of JEV were generated and analyzed. Both were efficiently expressed from both 5' and 3' dsSIN vectors. The 80%E product was efficiently secreted from both vertebrate and

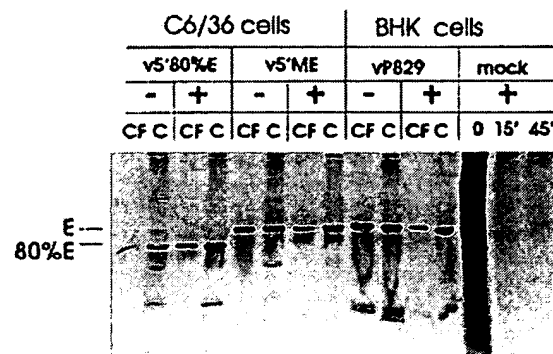


FIG. 9. Effect of cycloheximide treatment on secretion of E. C6/36 cells infected with v5'80%E or v5'ME or vP829-infected BHK cells were pulse-labeled 16 hr postinfection with [ $^{35}$ S]Met for 20 min and chased for 6 hr in maintenance medium (–) or maintenance medium containing 50  $\mu$ g/ml cycloheximide (+). Equal fractions of the cell lysates (C) or culture fluids (CF) were immunoprecipitated with an anti-E MAb and analyzed electrophoretically. To determine the efficiency of cycloheximide treatment, mock-infected BHK cell monolayers were incubated in culture medium containing 50  $\mu$ g/ml cycloheximide for 0, 15, and 45 min prior to 15 min labeling with [ $^{35}$ S]Met in medium lacking cycloheximide and equal fractions of the cell lysates were analyzed by SDS-PAGE, as shown in the right three lanes.



mosquito cells infected with the dsSIN recombinants, as expected since this protein lacks the membrane anchor sequence. This result is consistent with observations of efficient secretion of truncated forms of dengue virus E protein (Men *et al.*, 1991). In contrast, prM and E expressed by the dsSIN-prM-E constructs were secreted poorly from infected vertebrate cells (BHK, Vero, HeLa). In comparison, prM and E expressed by vaccinia virus recombinant vP829 that contained an identical prM-E cassette were efficiently secreted from infected BHK cells. Mosquito C6/36 cells infected with the dsSIN-prM-E recombinants efficiently secreted prM and E. As was found with vP829, prM and E secreted from both vertebrate and mosquito cells infected with dsSIN-prM-E recombinants were in the form of subviral particles.

The low level of secretion of subviral particles observed in mammalian cells infected with dsSIN-prM-E was due to a suppressive effect exerted by the dsSIN vectors since superinfection of vP829-infected cells with SIN reduced secretion of subviral particles. The suppressive effect was due to the SIN nonstructural proteins since secretion of prM and E expressed by a SIN replicon containing only the SIN nonstructural ORF was also inefficient. The suppression of subviral particle secretion is presumably correlated with the deleterious effect exerted on vertebrate cells by alphavirus replication. Consistent with this hypothesis, subviral particle secretion was suppressed to a greater degree late in infection compared to early in infection and subviral particle secretion was efficient in mosquito cells, in which no deleterious effect associated with alphavirus replication occurs (Wengler, 1980; Brown and Condreay, 1986). Studies on the SIN replicon have shown that it is as efficient as is complete virus in inhibiting vertebrate cell protein synthesis; however, timely development of microscopically observable CPE requires expression of the virus glycoproteins (which are absent in the SIN replicon) on the cytoplasmic membrane (Frolov and Schlesinger, 1994). This indicates that suppression of JEV subviral particle formation is caused by inhibition of cell protein synthesis. Consistent with this conclusion, VV recombinants that express the SIN nonstructural ORF did not suppress secretion of subviral particles expressed by vP829; these VV recombinants also did not inhibit VV-specific protein synthesis, as did SIN. [In explanation of this observation, it is possible that amplification of SIN genomic RNA (but not the subgenomic RNA; Frolov and Schlesinger, 1994) mediated by the SIN nonstructural proteins rather than the nonstructural proteins themselves are responsible for inhibition of cell protein synthesis by SIN (SIN RNA is amplified in replicon-infected cells but not in cells infected with vNS1234 or vNS1  $\times$  2  $\times$  3  $\times$  4(s))].

If caused by the inhibition of cell protein synthesis, the suppression of extracellular subviral particle production is presumably due to inhibition of synthesis of a labile cellular factor necessary for either subviral particle formation or secretion. This factor would be specific for JEV

subviral particle production since SIN virion formation is obviously ongoing in dsSIN-prM-E-infected cells and the secretion of individual proteins such as 80%E is also unaffected [additionally, dsSIN recombinants that express JEV NS1 efficiently secrete NS1 oligomers (K. V. Pugachev, unpublished observations)]. Interestingly, subviral particles were efficiently secreted from cells infected with vP829 even though VV has a profound effect on cell macromolecular synthesis (Moss, 1990). An experiment was done to simulate inhibition of protein synthesis by treating vP829-infected BHK cells and dsSIN-prM-E-infected C6/36 cells with cycloheximide after a pulse label, but no suppression of secretion of E was observed. However, the conditions of inhibition of cell protein synthesis in this experiment do not precisely mimic those in SIN-infected cells in which protein synthesis inhibition was ongoing from the initiation of infection until the pulse labeling. Therefore, there is a possibility that inhibition of protein synthesis per se may not exert a suppressive effect on subviral particle formation. In addition to its effect on protein synthesis, SIN infection modifies plasma membrane ion pump activity and changes the fatty acid composition of cellular membranes (Wengler, 1980; Brown and Condreay, 1986); either of these effects could reduce the secretion of subviral particles. However, the SIN-specific moieties responsible for these effects have not been determined.

Two points of general interest were raised by the data presented in this report. First, alphavirus expression vectors, which are gaining in popularity (Bredenbeek and Rice, 1992), promote synthesis of high levels of foreign proteins, as shown by the abundant expression of JEV proteins in dsSIN-JEV-infected and SIN-JEV replicon-transfected cells. SIN recombinants expressing JEV prM-E cassette produce subviral particles similar to those produced by cells infected with vP829 recombinant vaccinia virus. However, since interference with secretion of these particles occurred, normal processing of proteins expressed by alphavirus vectors, particularly glycoproteins, needs to be ascertained. Vaccination with vP829 induces protective immunity in mice to lethal challenge with neurovirulent strain of JEV and it has been shown that production of subviral particles is important for the protective effect (Konishi *et al.*, 1991, 1992). Whether the suppression of subviral particle formation will interfere with protective effect induced in mice with dsSIN-JEV recombinants remains to be determined. A second consideration in the use of alphavirus expression vectors is formation of self-replicating, self-assembling particles when structural proteins of other viruses are expressed in replicon vectors, a phenomenon which was recently demonstrated by Rolls *et al.* (1994) with SFV replicons that expressed the G protein of VSV. We tested SIN-prM-E replicons for the formation of similar infectious particles and were unable to detect any evidence for self-packaging of these types of replicons. Thus, formation of such self-replicating particles is dependent upon which

viral glycoprotein is expressed. Third, we assessed the replication of SIN and VV in coinfecting cells. It was previously shown that replication of SIN as assayed by production of PFU was unaffected by coinfection with VV (Rice *et al.*, 1985; Li *et al.*, 1991). We found that both viruses replicated since proteins expressed by both viruses were detected in every cell in the coinfecting culture. However, while total SIN protein synthesis was unaffected, VV protein synthesis was decreased fivefold. Thus, SIN has an inhibitory effect on the efficiency of expression of VV proteins while VV has no effect on SIN gene expression. This finding needs to be taken into consideration in experiments in which cells are coinfecting with VV- and SIN-recombinants.

The second point of general interest is that the interference with JEV subviral particle secretion by the dsSIN vectors could be reflective of a mechanism of competitive advantage on the part of alphaviruses in cells coinfecting with an alphavirus and a flavivirus exerted at the level of flavivirion formation. Relationships between JEV and SIN have not been studied directly in cell culture or experimental animals. However, Oaten *et al.* (1976) demonstrated that significant protection to heterologous intracerebral challenge with a flavivirus Langat occurred after intracerebral infection of mice with SFV and SIN and proposed that interference with Langat virus replication by SFV and SIN was the most important factor in protecting the mice from a lethal Langat virus encephalitis. Although alphaviruses and flaviviruses are taxonomically unrelated, they share an ecological niche. Other examples of interference between unrelated viruses that share ecological niches are the interference with hepatitis B infection exerted by hepatitis C virus (which apparently is due to the hepatitis C core protein) (Shih *et al.*, 1993; Fong *et al.*, 1991; Liaw *et al.*, 1991) and with adenovirus and herpesvirus oncogenicity and replication exerted by adeno-associated virus (reviewed by Berns, 1990). If specific mechanisms of heterologous virus interference could be elucidated, it could potentially lead to development of new antiviral strategies.

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## REFERENCES

- Berns, K. I. (1990). Parvoviridae and their replication. In "Fields Virology II." (B. N. Fields and D. A. Knipe, Eds.), Vol. 2, pp. 1743–1763. Raven Press, New York.
- Bray, M., Zhao, B., Markoff, L., Eckels, K. H., Chanock, R. M., and Lai, C.-J. (1989). Mice immunized with recombinant vaccinia virus expressing dengue 4 virus structural proteins with or without nonstructural protein NS1 are protected against fatal dengue virus encephalitis. *J. Virol.* 63, 2853–2856.
- Bredenbeek, P. J., and Rice, C. M. (1992). Animal RNA virus expression systems. *Semin. Virol.* 3, 297–310.
- Bredenbeek, P. J., Frolov, I., Rice, C. M., and Schlesinger, S. (1993). Sindbis virus expression vectors: Packaging of RNA replicons by using defective helper RNAs. *J. Virol.* 67, 6439–6446.
- Brown, D. T., and Condeelis, L. D. (1986). Replication of alphaviruses in mosquito cells. In "The Togaviridae and Flaviviridae" (S. Schlesinger and M. J. Schlesinger, Eds.), pp. 171–207. Plenum, New York/London.
- Chambers, T. J., Hahn, C. S., Galler, R., and Rice, C. M. (1990). Flavivirus genome organization, expression and replication. *Annu. Rev. Microbiol.* 44, 649–688.
- Davis, N. L., Willis, L., Smith, J. F., and Johnston, R. E. (1989). *In vitro* synthesis of infectious Venezuelan equine encephalitis virus RNA from a cDNA clone: Analysis of a viable deletion mutant. *Virology* 171, 189–204.
- Fong, T.-L., DiBisceglie, A. M., Waggoner, J. G., Banks, S. M., and Hoofnagle, J. H. (1991). The significance of antibody to hepatitis C virus in patients with chronic hepatitis B. *Hepatology* 14, 64–67.
- Frey, T. K. (1994). Molecular biology of rubella virus. *Adv. Virus Res.* 44, 69–160.
- Frolov, I., and Schlesinger, S. (1994). Comparison of the effects of Sindbis virus and Sindbis virus replicons on host cell protein synthesis and cytopathogenicity in BHK cells. *J. Virol.* 68, 1721–1727.
- Fuerst, T. R., Niles, E. G., Studier, F. W., and Moss, B. (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* 83, 8122–8126.
- Hahn, C. S., Hahn, Y. S., Braciale, T. J., and Rice, C. M. (1992). Infectious Sindbis virus transient expression vectors for studying antigen processing and presentation. *Proc. Natl. Acad. Sci. USA* 89, 2679–2683.
- Heinz, F. X., and Roehrig, J. T. (1990). Flaviviruses. In "Immunochimistry of Viruses. II. The Basis for Serodiagnosis and Vaccines" (M. H. V. van Regenmortel and A. R. Neurath, Eds.), pp. 289–305. Elsevier, Amsterdam.
- Konishi, E., Pincus, S., Fonseca, B. A. L., Shope, R. E., Paoletti, E., and Mason, P. W. (1991). Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. *Virology* 185, 401–410.
- Konishi, E., Pincus, S., Paoletti, E., Shope, R. E., Burrage, T., and Mason, P. W. (1992). Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. *Virology* 188, 714–720.
- Kuhn, R. J., Niesters, H. G. M., Hong, Z., and Strauss, J. H. (1991). Infectious RNA transcripts from Ross River virus cDNA clones and the construction and characterization of defined chimeras with Sindbis virus. *Virology* 182, 430–441.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lemm, J. A., and Rice, C. M. (1993). Roles of nonstructural polyproteins and cleavage products in regulating Sindbis virus RNA replication and transcription. *J. Virol.* 67, 1916–1926.
- Li, G., Prágai, B. M., and Rice, C. M. (1991). Rescue of Sindbis virus-specific RNA replication and transcription by using a vaccinia virus recombinant. *J. Virol.* 65, 6714–6723.
- Liaw, Y.-F., Lin, S. M., Sheen, I. S., and Chu, C. M. (1991). Acute hepatitis C virus superinfection followed by spontaneous HBsAg seroconversion and HBsAg elimination. *Infection* 250, 54–55.
- Liljeström, P., and Garoff, H. (1991). A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Bio/Technology* 9, 1356–1361.
- Liljeström, P., Lusa, S., Huylebroeck, D., and Garoff, H. (1991). *In vitro* mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. *J. Virol.* 65, 4107–4113.
- Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Mason, P. W., McAda, P. C., Dalrymple, J. M., Fournier, M. J., and Mason, T. L. (1987). Expression of Japanese encephalitis virus antigens in *Escherichia coli*. *Virology* 158, 361–372.

- Mason, P. W. (1989). Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology* 169, 354-364.
- McAda, P. C., Mason, P. W., Schmaljohn, C. S., Dalrymple, J. M., Mason, T. L., and Fournier, M. J. (1987). Partial nucleotide sequence of the Japanese encephalitis virus genome. *Virology* 158, 348-360.
- Men, R., Bray, M., and Lai, C.-J. (1991). Carboxy-terminally truncated dengue virus envelope glycoproteins expressed on the cell surface and secreted extracellularly exhibit increased immunogenicity in mice. *J. Virol.* 65, 1400-1407.
- Monath, T. P. (1986). Pathobiology of the flaviviruses. In "The Togaviridae and Flaviviridae" (S. Schlesinger and M. J. Schlesinger, Eds.), pp. 375-440. Plenum, New York/London.
- Moss, B. (1990). Poxviridae and their replication. In "Fields Virology II." (B. N. Fields and D. A. Knipe, Eds.), Vol. 2, pp. 2079-2111. Raven Press, New York.
- Oaten, S. W., Webb, H. E., and Bowen, E. T. W. (1976). Enhanced resistance of mice to infection with Langat (TP21) virus following pre-treatment with Sindbis or Semliki Forest virus. *J. Gen. Virol.* 33, 381-388.
- Raju, R., and Huang, H. V. (1991). Analysis of Sindbis virus promoter recognition in vivo, using novel vectors with two subgenomic mRNA promoters. *J. Virol.* 65, 2501-2510.
- Rice, C. M., Franke, C. A., Strauss, J. H., and Hruby, D. E. (1985). Expression of Sindbis virus structural proteins via recombinant vaccinia virus: Synthesis, processing, and incorporation into mature Sindbis virions. *J. Virol.* 56, 227-239.
- Rice, C. M., Lewis, R., Strauss, J. H., and Huang, H. V. (1987). Production of infectious RNA transcripts from Sindbis virus cDNA clones: mapping of lethal mutations, rescue of temperature-sensitive marker, and in vitro mutagenesis to generate defined mutants. *J. Virol.* 61, 3809-3819.
- Rolls, M. M., Webster, P., Balba, N. H., and Rose, J. K. (1994). Novel infectious particles generated by expression of the vesicular stomatitis virus glycoprotein from a self-replicating RNA. *Cell* 79, 497-506.
- Schlesinger, S. (1993). Alphaviruses - Vectors for the expression of heterologous genes. *Trends Biotechnol.* 11, 18-22.
- Shih, G.-M., Lo, S. J., Miyamura, T., Chen, S.-Y., and Lee, Y.-H. W. (1993). Suppression of hepatitis B virus expression and replication by hepatitis C virus core protein in Huh-7 cells. *J. Virol.* 67, 5823-5832.
- Shope, R. E. (1980). Medical significance of togaviruses: An overview of diseases caused by togaviruses in man and domestic and wild vertebrate animals. In "The Togaviruses" (R. W. Schlesinger, Ed.), pp. 47-82. Academic Press, New York/London.
- Stern, W., and Dales, S. (1974). Biogenesis of vaccinia: Concerning the origin of the envelope phospholipids. *Virology* 62, 293-306.
- Strauss, J. H., and Strauss, E. G. (1994). The alphaviruses: Gene expression, replication and evolution. *Microbiol. Rev.* 58, 491-562.
- Tesh, R. B. (1979). A method for the isolation and identification of dengue viruses, using mosquito cell cultures. *Am. J. Trop. Med. Hyg.* 28, 1053-1059.
- Wengler, G. (1980). Effects of alphaviruses on host cell macromolecular synthesis. In "The Togaviruses: Biology, Structure, Replication" (R. W. Schlesinger, Ed.), pp. 459-472. Academic Press, New York.
- Xiong, C., Lewis, R., Shen, P., Schlesinger, S., Rice, C. M., and Huang, H. V. (1989). Academic Press, New York. Sindbis virus: An efficient, broad host range vector for gene expression in animal cells. *Science* 243, 1188-1191.

## Induction of Protective Immunity against Japanese Encephalitis in Mice by Immunization with a Plasmid Encoding Japanese Encephalitis Virus Premembrane and Envelope Genes

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A DNA vaccine plasmid containing the Japanese encephalitis (JE) virus premembrane (prM) and envelope (E) genes (designated pcDNA3JEME) was evaluated for immunogenicity and protective efficacy in mice. Two immunizations of 4-week-old female ICR mice with pcDNA3JEME by intramuscular or intradermal injections at a dose of 10 or 100 µg per mouse elicited neutralizing (NEUT) antibodies at titers of 1:10 to 1:20 (90% plaque reduction), and all immunized mice survived a challenge with 10,000 50% lethal doses of the P3 strain of JE virus. A single immunization with 100 µg of pcDNA3JEME did not elicit detectable NEUT antibodies but induced protective immunity. Spleen cells obtained from BALB/c mice immunized once with 10 or 100 µg of pcDNA3JEME contained JE virus-specific memory cytotoxic T lymphocytes (CTLs). BALB/c mice maintained detectable levels of memory B cells and CTLs for at least 6 months after one immunization with pcDNA3JEME at a dose of 100 µg. The CTLs induced in BALB/c mice immunized twice with 100 µg of pcDNA3JEME were CD8 positive and recognized mainly the envelope protein. These results indicate that pcDNA3JEME has the ability to induce a protective immune response which includes JE virus-specific antibodies and CTLs.

One of the recent promising strategies in protection from viral diseases is the induction of protective immunity by the expression of subsets of viral genes in the vaccinated host. This strategy can eliminate immune responses to unneeded or adventitious antigens present in inactivated virus vaccine preparations and may provide improved safety relative to live attenuated virus vaccines. The introduction of subsets of viral genes into a vaccinee can be accomplished with a recombinant virus (32) or with naked DNA molecules designed to express the genes in the cells of the host (22).

We have studied Japanese encephalitis (JE) as a model for understanding the immunogenicity and protective efficacy conferred on murine, porcine, and human hosts by different flavivirus gene products. In these studies, we showed that recombinant poxviruses carrying the signal sequence for the pre-membrane (prM), the prM gene, and the envelope (E) gene express proper forms of the prM and E proteins in infected cells and that infected cells release these viral proteins in a particulate form (15, 25). These extracellular particles are morphologically and biochemically similar to the authentic subviral particles, so-called slowly sedimenting hemagglutinin, released from JE virus-infected cells (17). The similarity of these genetically engineered products to natural virus particles is consistent with our early work showing the excellent performance of vaccinia virus-based vaccines specific for these particles in mice (15, 25). Furthermore, a recombinant poxvirus carrying the same signal sequence-prM-E cassette but based on a highly

attenuated vaccinia virus strain (NYVAC) induced high levels of neutralizing (NEUT) antibodies (16) and specific cytotoxic T lymphocytes (CTLs) in mice (13) and protected mice from lethal challenge and swine from viremia (16). However, this NYVAC-based recombinant poxvirus did not induce NEUT antibodies to JE virus in vaccinia virus-preimmune vaccinees in a clinical phase I trial, although it did elicit anti-JE virus antibodies in vaccinia virus-naïve vaccinees (14).

The adverse effect of antivector immunity to the immunogenicity of the products specified by the vector has been pointed out with several systems (2, 8, 33) and may cause significant problems for the viral vector-based strategy, especially in long-lived species, such as humans. Naked DNA vaccines, which do not suffer from the problem of antivector immunity, recently have been developed and tested for a variety of viral pathogens (3, 31, 34–36). Recently, naked DNA vaccine candidates have been reported for two flavivirus diseases. Work with St. Louis encephalitis showed that a plasmid carrying the prM and E genes could induce partial protection in mice, but induction of NEUT antibodies and CTLs was not demonstrated (28). Another plasmid containing the prM gene and part of the E gene of dengue type 2 virus induced NEUT antibodies, but protection was not demonstrated (12). In this report, we studied the immunogenicity and protective efficacy of plasmid DNA containing the signal sequence-prM-E cassette of JE virus genes that we had identified to be the most effective immunogen in poxvirus-based recombinant viral vaccines for JE.

### MATERIALS AND METHODS

**Construction of plasmids.** The JE virus cDNA containing the prM signal sequence, the prM gene, and the E gene was amplified by PCR with DNA template plasmid pARJa (containing Nakayama strain C protein cDNA sequences fused to plasmids PM-7 and PM-6 [26]; GenBank accession no. M73710). The sense

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primer included an *EcoRI* site, an efficient eukaryotic initiation site (19), and a start codon, followed by the codons encoding Glu-Gly-Ser of the prM signal sequence. The antisense primer corresponded to the C-terminal six codons of the E gene, a termination codon, and an *XhoI* site. To facilitate "error-free" amplification, the selected JE virus coding region was amplified in two portions, which were combined by use of an artificial *EcoRV* site that was added within the coding region (codons 67 and 68 of the E protein) without changing the encoded amino acid sequence. The amplified cDNA was inserted into the pcDNA3 vector (Invitrogen Corp., San Diego, Calif.) at the *EcoRI/XhoI* site between the strong eukaryotic promoter derived from human cytomegalovirus and the polyadenylation signal derived from the bovine growth hormone. The construct was designated pcDNA3JEME. Proper insertion of the gene cassette in pcDNA3JEME was confirmed by sequencing with a DNA sequencer (ABI 373A; Applied Biosystems, Chiba, Japan). pcDNA3JEME DNA was purified with a Qiagen Plasmid Kit (Funakoshi Co. Ltd., Tokyo, Japan) following the manufacturer's instructions and was used for immunization of mice.

**Viruses.** The prototype Nakayama strain of JE virus (23) was used in all *in vitro* studies, including NEUT tests, spleen cell stimulation, and cytotoxicity assays. The Nakayama strain, which exhibits low lethality in many strains of mice, was also used to "vaccinate" mice. The Beijing P3 strain of JE virus, which is reproducibly virulent in mice over 6 weeks of age, was used for mouse challenge studies (25). Recombinant vaccinia viruses used for infection of target cells in cytotoxicity assays were vP555, carrying the prM, E, and NS1 genes of the Nakayama strain of JE virus; vP658, carrying the E and NS1 genes; vP829, carrying the prM and E genes; and their parent virus, vP410 (15).

**Mouse experiments.** Groups of five 4-week-old female ICR mice were used for evaluating the induction of NEUT antibodies and protective immunity, and groups of two 6-week-old male BALB/c mice were used mainly for evaluating the induction of CTLs. Mice were immunized with pcDNA3JEME at doses of 0.1 to 100 µg, pcDNA3 at a dose of 100 µg, or phosphate-buffered saline (PBS) once or twice at an interval of 2 weeks. The injection route was intramuscular (i.m.) at both thighs or intradermal (i.d.) at the base of the tail. At 2 or 3 weeks after immunization, the ICR mice were bled retro-orbitally, and serum samples were isolated from blood, pooled, and used for evaluation of antibody. The ICR mice were also challenged by intraperitoneal (i.p.) injection with 10,000 50% lethal doses (LD<sub>50</sub>) of the P3 strain of JE virus and observed for 3 weeks. Postchallenge blood was collected from mice that survived the challenge. Spleen cell suspensions were prepared from BALB/c mice as previously described (13) and stimulated with JE virus for cytotoxicity assays (see below). For examination of the duration of NEUT antibodies and memory B cells and CTLs, BALB/c mice that had received one inoculation with pcDNA3JEME at a dose of 100 µg were kept for 1 to 6 months before sample collection.

**NEUT tests.** Specific antibodies elicited in immunized mice were evaluated by NEUT tests as previously described (15). The NEUT titer was expressed as the serum dilution yielding a 90% reduction in plaque number.

**Cytotoxicity assays.** Stimulation of spleen cells with JE virus *in vitro* and cytotoxicity assays were performed as previously described (13) with some modifications. Spleen cells ( $4 \times 10^6$ ) were stimulated by incubation with live JE virus antigen at a final dilution of 1:8 in 2 ml of RPMI 1640 medium containing 10% fetal bovine serum (RPMI-10% FBS) per well of 24-well microplates at 37°C for 6 days. The live virus antigen used was clarified culture fluid harvested from infected C6/36 cell cultures and contained a titer of approximately  $2 \times 10^8$  PFU/ml in the undiluted stock, as titrated on Vero cell cultures. The control antigen used was culture fluid from mock-infected C6/36 cell cultures. Both live virus and control antigens were used at 1:8 dilutions. Following the 6-day stimulation step, the cells were washed three times with RPMI-10% FBS and distributed in triplicate in 96-well microplates at different cell densities to provide various effector/target (E/T) ratios. The target cells used for these assays were primary mouse kidney (PMK) cells prepared from kidneys of BALB/c mice or P815 mastocytoma cells. PMK cells were infected with JE virus at a high multiplicity of infection (approximately 100 to 200 PFU/cell) or mock infected 17 to 18 h before the assay, and P815 cells were infected with vP829 or vP410 at a multiplicity of infection of 10 PFU/ml or mock infected 15 to 20 h before the assay. For target protein analysis, P815 cells infected with vP555 and vP658 were also used. All target cells were labeled with Na<sup>51</sup>CrO<sub>4</sub>, washed, and distributed evenly at  $1 \times 10^3$  or  $2 \times 10^3$  viable cells per well into microplates containing effector cells. The plates were incubated for 5 to 6 h at 37°C, and <sup>51</sup>Cr release into the supernatant was measured in a gamma counter. Percent specific lysis was calculated with the following formula:  $100 \times [(\text{experimental release} - \text{minimum release}) / (\text{maximum release} - \text{minimum release})]$ ; the maximum release was obtained by lysing all the target cells with Renex, and the minimum release was obtained with target cells incubated alone in RPMI-10% FBS.

**Cell depletion assays.** Cell depletion tests were performed as previously described (13). Briefly, cells stimulated with JE virus were incubated with antibodies to CD3, CD4, and CD8 at dilutions of 1:5 to 1:100 at 4°C for 30 min. These cells were then treated with rabbit complement at a 1:10 dilution at 37°C for 1 h and used in cytotoxicity assays. Cytotoxicity was compared with that obtained with JE virus-stimulated cells treated only with complement and with JE virus-stimulated and mock-stimulated cells without treatment.

TABLE 1. Immunogenicity of pcDNA3JEME in ICR mice with two immunizations

Immunogen <sup>a</sup>	Route	Dose at wk <sup>b</sup>		NEUT titer <sup>c</sup> at wk <sup>b</sup>			Survival <sup>d</sup>
		4	6	6	8	11	
pcDNA3JEME	i.m.	100 µg	100 µg	<1:10	1:20	1:640	5/5
pcDNA3JEME	i.m.	10 µg	10 µg	<1:10	1:10	1:320	5/5
pcDNA3JEME	i.d.	100 µg	100 µg	<1:10	1:10	1:160	5/5
pcDNA3JEME	i.d.	10 µg	10 µg	<1:10	1:20	1:640	5/5
PBS	i.m.			<1:10	<1:10	NA <sup>e</sup>	0/5
JE virus <sup>f</sup>	i.p.	$5 \times 10^6$ PFU	None	1:80	1:160	$\geq 1:1,280$	2/2

<sup>a</sup> Each immunogen was given to five female ICR mice at the ages indicated, and the mice were challenged at week 8.

<sup>b</sup> Ages of the mice at the time of inoculations or serum collections; 11 weeks indicates 3 weeks postchallenge.

<sup>c</sup> Represented as the serum dilution yielding a 90% reduction in plaque number.

<sup>d</sup> Number of surviving mice/total number of mice tested 3 weeks after challenge with 10,000 LD<sub>50</sub> of the P3 strain of JE virus.

<sup>e</sup> NA, serum was not available.

<sup>f</sup> The immunogen given at 4 weeks consisted of the live Nakayama strain of JE virus at a dose that killed three of five mice. Therefore, all serological and challenge data were derived from the remaining two mice.

## RESULTS

**Proper expression of pcDNA3JEME.** COS7 and Vero cells were transfected with pcDNA3JEME by use of liposomes (Lipofectin; Life Technologies Inc., Gaithersburg, Md.) or lipopolyamine (Transfectam; Biosepra, Villeneuve-la-Garenne, France), and expression was determined by indirect fluorescent-antibody staining with a monoclonal antibody to the JE virus E protein (J3-11B9) (24). When transfection protocols recommended by the manufacturer were used, E antigen could be detected with this antibody in 3 to 5% of either COS7 or Vero cells 1 to 2 days after transfection, indicating that pcDNA3JEME expressed the E antigen in these eukaryotic cells.

**Induction of NEUT antibodies and protective immunity.** ICR mice were inoculated i.m. or i.d. with 10 or 100 µg of pcDNA3JEME at 4 and 6 weeks of age (Table 1). At 2 weeks after each immunization (at 6 and 8 weeks of age), sera were collected and checked for NEUT antibodies. Following the second serum collection, these mice were challenged with 10,000 LD<sub>50</sub> of the P3 strain of JE virus and observed for 3 weeks; sera were collected at the end of the 3-week observation period from all mice that survived the challenge (at 11 weeks of age). The results in Table 1 show that one immunization with pcDNA3JEME did not induce detectable levels of NEUT antibodies but that two immunizations induced NEUT antibodies at titers of 1:10 to 1:20, irrespective of the immunization route and the dose. All mice given two inoculations survived the challenge but displayed significantly increased serum NEUT titers (1:160 to 1:640) at 3 weeks postchallenge. Mice inoculated with PBS did not have detectable levels of NEUT antibodies and died from the challenge, whereas mice vaccinated by inoculation with the Nakayama strain of JE virus 4 weeks prior to challenge had a high NEUT antibody titer and survived the P3 virus challenge. Since three of five mice died following infection with  $5 \times 10^6$  PFU of the Nakayama strain of JE virus, this "vaccinating" dose corresponded to approximately 1 LD<sub>50</sub>. These results indicate that two immunizations with pcDNA3JEME induced NEUT antibodies and protective immunity in mice. Since a significant difference in NEUT antibody titer and survival rate was not observed between the i.m. and i.d. routes, we used the i.m. route for subsequent experiments.

Next, the ability of pcDNA3JEME to induce protective im-

TABLE 2. Immunogenicity of pcDNA3JEME in ICR mice with one immunization

Immunogen <sup>a</sup>	Route	Dose ( $\mu$ g) at 4 wk <sup>b</sup>	NEUT titer <sup>c</sup> at wk <sup>b</sup>		Survival <sup>d</sup>
			7	10	
pcDNA3JEME	i.m.	100	<1:10	1:160	4/5
pcDNA3JEME	i.m.	10	<1:10	NA <sup>e</sup>	0/5
pcDNA3JEME	i.m.	1	<1:10	NA	0/5
pcDNA3JEME	i.m.	0.1	<1:10	NA	0/5
pcDNA3	i.m.	100	<1:10	NA	0/5
PBS	i.m.		<1:10	NA	0/5

<sup>a</sup> Each immunogen was given to five female ICR mice at 4 weeks of age, and the mice were challenged at week 7.

<sup>b</sup> Ages of the mice at the time of inoculations or serum collections; 10 weeks indicates 3 weeks postchallenge.

<sup>c</sup> Represented as the serum dilution yielding a 90% reduction in plaque number.

<sup>d</sup> Number of surviving mice/total number of mice tested 3 weeks after challenge with 10,000 LD<sub>50</sub> of the P3 strain of JE virus.

<sup>e</sup> NA, serum was not available.

munity in ICR mice was examined in the one-immunization protocol at 0.1 to 100  $\mu$ g (Table 2). Immunization was performed at 4 weeks of age, with bleeding and challenge at 7 weeks, observation for 3 weeks, and postchallenge bleeding when the mice were 10 weeks old. Nonimmune control groups included mice inoculated with PBS or pcDNA3 at 100  $\mu$ g. All mice immunized with pcDNA3JEME at 0.1 to 10  $\mu$ g, pcDNA3, and PBS died from challenge, but partial protection was observed for mice immunized with pcDNA3JEME at 100  $\mu$ g. NEUT antibodies were not observed for prechallenge sera for any of these groups, but a high NEUT antibody titer was observed for surviving mice immunized with 100  $\mu$ g of pcDNA3JEME.

**Induction of specific CTLs.** In order to study cellular responses in mice immunized with pcDNA3JEME, spleen cells were obtained from BALB/c mice immunized with pcDNA3JEME at 100  $\mu$ g twice at a 2-week interval, stimulated in vitro with JE virus, and examined for cytotoxic activity against PMK cells infected with JE virus. Figure 1 shows the results obtained at an E/T ratio of 100:1. A high percentage of specific lysis of JE virus-infected cells (approximately 50%) was obtained with effector cells stimulated with JE virus but not with unstimulated effector cells. Cytotoxic activities against mock-infected cells were low. This result indicates that immunization with pcDNA3JEME induced JE virus-specific memory CTLs in mice.

The ability of pcDNA3JEME to induce specific CTLs in BALB/c mice was examined in the one-immunization protocol

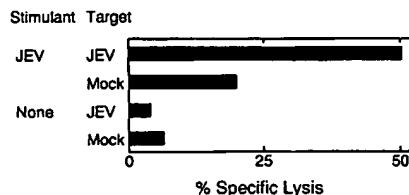


FIG. 1. Lysis of JE virus-infected PMK cells by pcDNA3JEME-immune spleen cells stimulated with JE virus. BALB/c mice were immunized with pcDNA3JEME at 100  $\mu$ g twice at an interval of 2 weeks. At 2 weeks after the second immunization, the spleen cells were harvested and stimulated by incubation with C6/36-grown virus (JEV) or culture fluid from uninfected C6/36 cells (None) for 6 days. Cytotoxic activities against JE virus-infected (JEV) or mock-infected (Mock) PMK cells were measured at an E/T ratio of 100:1 by the standard chromium release method (see Materials and Methods for details).

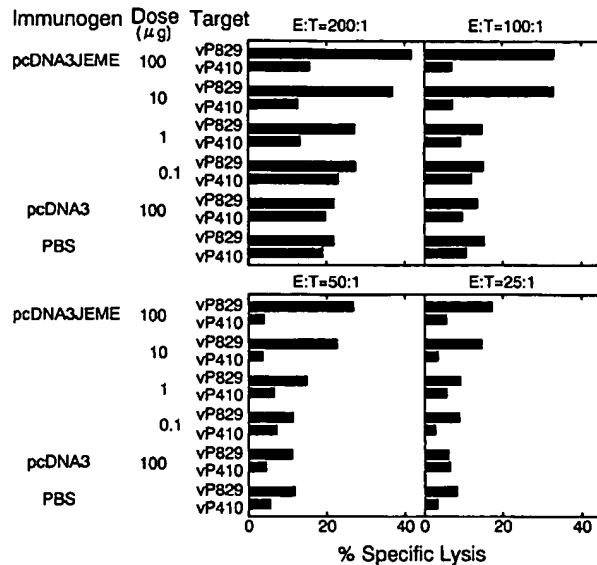


FIG. 2. Lysis of P815 cells infected with a recombinant vaccinia virus carrying the prM and E genes (vP829) or the parental virus (vP410) by pcDNA3JEME-immune spleen cells stimulated with JE virus. BALB/c mice were immunized once with pcDNA3JEME at 0.1 to 100  $\mu$ g, pcDNA3 at 100  $\mu$ g, or PBS. At 3 weeks postimmunization, the spleen cells were harvested and incubated with C6/36-grown virus for 6 days. Cytotoxic activities were measured at the indicated E/T ratios by the standard chromium release method (see Materials and Methods for details).

at 0.1 to 100  $\mu$ g (Fig. 2). Specific cytotoxic activities were observed for mice immunized with 10 or 100  $\mu$ g of pcDNA3JEME. However, responses in mice immunized with 1 or 0.1  $\mu$ g of this DNA were not significantly different from the responses detected in samples collected from mice inoculated with 100  $\mu$ g of pcDNA3 or PBS.

**Characterization of CTLs.** The phenotype of cells responsible for cytotoxic activities was determined by use of cell depletion tests (Fig. 3). Spleen cells obtained from BALB/c mice immunized twice with 100  $\mu$ g of pcDNA3JEME and stimulated in vitro with JE virus were treated with antibodies against cell surface markers and complement before cytotoxicity assays. Cytotoxic activities were reduced by treatment with anti-CD3 or anti-CD8 in the presence of complement, whereas treatment with complement alone or with anti-CD4 and complement did not reduce the activities. These results indicate that

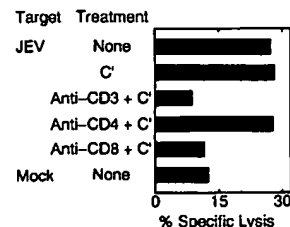


FIG. 3. Phenotypic analysis of CTLs by cell depletion with the indicated antibodies and complement. BALB/c mice were immunized with pcDNA3JEME at 100  $\mu$ g twice at an interval of 2 weeks. Four weeks later, the spleen cells were harvested and incubated with C6/36-grown virus for 6 days. After cell depletion (see Materials and Methods for details), cytotoxic activities were measured at an E/T ratio of 120:1 with <sup>51</sup>Cr-labeled P815 cells infected with vP829. JEV, JE virus; Mock, mock infected.

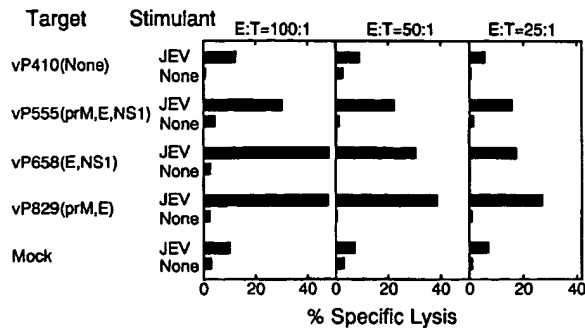


FIG. 4. Target antigen analysis of CTLs. BALB/c mice were immunized with pcDNA3JEME at 100  $\mu$ g twice at an interval of 2 weeks. At 3 weeks later, the spleen cells were harvested and incubated with C6/36-grown virus for 6 days. Cytotoxic activities were measured at the indicated E/T ratios with  $^{51}$ Cr-labeled P815 cells infected with a recombinant vaccinia virus encoding no antigens (parental virus vP410), prM, E, and NS1 (vP555), E and NS1 (vP658), or prM and E (vP829) or mock infected (Mock). JEV, JE virus.

CD8-positive and CD4-negative T lymphocytes were responsible for cytotoxic activities.

We investigated JE virus proteins recognized by CTLs by using P815 cells infected with recombinant vaccinia viruses expressing different JE virus antigens (Fig. 4). Spleen cells obtained from BALB/c mice immunized twice with 100  $\mu$ g of pcDNA3JEME and stimulated in vitro with JE virus were used for this experiment. Specific cytotoxic activities were observed against target cells infected with vP555, vP658, and vP829; all of these recombinant viruses expressed E protein with or without prM protein. These results suggest that the predominant CTLs induced in pcDNA3JEME-immunized mice recognized E protein. Since target cells expressing prM protein alone were not used in this experiment, the presence or absence of a minor population of CTLs which specifically recognize prM protein could not be determined.

**Duration of immunity induced by pcDNA3JEME.** The levels of NEUT antibodies and protective immunity were examined with groups of two male BALB/c mice at 1 to 6 months after one immunization with pcDNA3JEME at 100  $\mu$ g. Since undetectable levels of NEUT antibodies were found in ICR mice 3 weeks after one immunization (at 7 weeks of age; Table 2), the BALB/c mice in this experiment testing duration of immunity were challenged at different intervals following immunization, and the induction of NEUT antibodies at 4, 8, and 21 days after challenge was used as an indicator of the presence of vaccination-induced memory B cells (Table 3). As expected, prior to challenge (day -2 in Table 3), only low or undetectable NEUT antibody titers were observed for each pair of mice immunized with pcDNA3JEME, and no NEUT antibodies were detectable in mice inoculated with PBS. Following challenge, the NEUT antibody titers in mice immunized with pcDNA3JEME were elevated to 1:160 to 1:640 by day 4 and to 1:320 to 1:1,280 by day 8, and these levels were maintained or further increased until day 21. On the other hand, the titers of NEUT antibodies in unimmunized mice remained low (undetectable or 1:10) until days 4 and 8. All unimmunized mice died before day 10, and all immunized mice survived throughout the observation period (21 days). Although only two mice were used per group in this experiment, the results indicate that mice immunized with pcDNA3JEME maintained sufficient memory B cells to supply high titers of NEUT antibodies if challenged within 6 months of immunization.

The levels of memory CTLs were examined with two

male BALB/c mice at 6 months after one immunization with pcDNA3JEME at 100  $\mu$ g. The percentages of specific lysis obtained against vP829- and vP410-infected targets were 39.3 and 13.6% at an E/T ratio of 400:1 and 23.9 and 5.4% at an E/T ratio of 200:1, respectively. This result indicates that mice immunized with pcDNA3JEME maintained detectable levels of memory CTLs for at least 6 months.

## DISCUSSION

This paper demonstrates that the JE virus prM and E genes introduced into mice in the form of plasmid DNA induced NEUT antibodies, CTLs, and protective immunity. In flavivirus infections, the prM, E, and NS1 proteins have been considered to induce protective immunity, since protection of mice from lethal challenge has been shown by passive transfer of monoclonal antibodies against the prM (10), E (11, 24) and NS1 (7) proteins. Recently, several epitopes on other viral proteins important for the induction of cellular immunity, including CTLs, were analyzed (5, 20, 21), but no epitopes were demonstrated to be responsible for protection. Although passive transfer of JE virus-specific CTLs protected mice from lethal challenge (27), the epitopes recognized by these CTLs were not characterized.

Our previous studies with poxvirus-based recombinant JE viruses demonstrated the proper synthesis of intracellular and extracellular forms of prM and E proteins in cells infected with recombinants encoding the signal sequence of prM, prM, and E, independent of the vector virus used: vaccinia virus (15, 16), canary poxvirus (18), and Sindbis virus (30). Furthermore, a similar cassette can be used to synthesize extracellular particles containing the structural proteins of other flaviviruses, including yellow fever virus (29), dengue type 1 virus (4), and tick-borne encephalitis virus (1). In the present study, we showed that a plasmid carrying these JE virus genes could be used to produce E protein in COS7 and Vero cells. Since the in vitro transfection efficiency of pcDNA3JEME was low in these cells, we did not attempt to identify extracellular forms of E in transfected cell cultures. However, the proper synthesis of E in the transfected cells was supported by the induction of specific

TABLE 3. Duration of immunity to pcDNA3JEME in BALB/c mice<sup>a</sup>

Interval between immunization and challenge (mo) <sup>b</sup>	Immunogen	NEUT titer <sup>c</sup> at day <sup>d</sup>				Survival <sup>e</sup>
		-2	4	8	21	
1	pcDNA3JEME	1:10	1:160	1:320	1:640	2/2
1	PBS	<1:10	1:10	1:10	NA <sup>f</sup>	0/2
2	pcDNA3JEME	<1:10	1:160	1:640	1:640	2/2
4	pcDNA3JEME	1:10	1:160	1:640	1:640	2/2
4	PBS	<1:10	1:10	NA	NA	0/2
6	pcDNA3JEME	1:10	1:640	1:1,280	≥1:1,280	2/2

<sup>a</sup> The duration was demonstrated by protection from challenge and anamnestic antibody response at up to 6 months following a single immunization.

<sup>b</sup> Groups of two male BALB/c mice were inoculated with PBS or 100  $\mu$ g of pcDNA3JEME at 6 weeks of age and then challenged 1, 2, 4, or 6 months later.

<sup>c</sup> Represented as the serum dilution yielding a 90% reduction in plaque number.

<sup>d</sup> Days relative to challenge. Day -2 indicates 2 days before challenge.

<sup>e</sup> Number of surviving mice/total number of mice tested 21 days after challenge with the same dose of the P3 strain of JE virus as was used for the challenge of ICR mice (Table 2).

<sup>f</sup> NA, serum was not available due to the death of mice. Two mice immunized with PBS 1 month earlier died on days 6 and 10, and two mice immunized with PBS 4 months earlier died on days 6 and 8.

antibodies, CTLs, and protective immunity in mice inoculated with pcDNA3JEME.

Several devices to increase the level of expression of JE virus-proteins were incorporated into pcDNA3JEME. We chose a vector with a strong eukaryotic promoter derived from human cytomegalovirus and a well-characterized polyadenylation signal derived from bovine growth hormone. We inserted a strong eukaryotic initiation site containing an ACC sequence which precedes the AUG start codon and which has been reported to be an optimal sequence for initiation by eukaryotic ribosomes (19). We also altered the prM signal sequence to enhance expression. Specifically, we added 5 amino acids to the 15-amino-acid sequence that we used in earlier poxvirus recombinants (15, 25), based on results which were obtained with recombinant vaccinia viruses encoding similar cassettes for other flavivirus genomes and which showed that longer prM signal sequences resulted in higher levels of synthesis of extracellular particles (data not shown). Furthermore, we did not include the gene for NS1 in pcDNA3JEME, since the production of extracellular particles (which we believe are the critical immunogens) from cells infected with vP829 carrying the prM and E genes was eight times higher than the production with vP555 carrying the prM, E, and NS1 genes and since vP829 induced higher levels of protective immunity than vP555 in mice (15).

Induction of CTLs is one of the prominent features of DNA immunization. Cumulative experimental data have established a theory that peptides expressed by foreign genes introduced into cells are bound to major histocompatibility complex class I molecules and are recognized by CD8-positive T lymphocytes, including CTLs (6). In the present study, memory CTLs were demonstrated in pcDNA3JEME-immunized BALB/c mice. CTLs induced by pcDNA3JEME immunization recognized mainly E protein, consistent with our previous data indicating that recombinant poxviruses carrying prM, E, and NS1 proteins induced CTLs that recognized mainly E protein (13). Interestingly, mice immunized once with 100 µg of pcDNA3JEME, which did not induce high levels of NEUT antibodies, were protected from lethal challenge. Immunization with the same dose of pcDNA3JEME induced CTLs in BALB/c mice, making it tempting to speculate that the protection observed in animals given a single dose of 100 µg was due to CTL responses. However, it is possible that low levels of antibodies (below the detection limit in our assay) present prior to challenge or antibodies produced by memory B cells and helper T cells that were rapidly activated following i.p. exposure to the challenge virus may have been responsible for protection. Current studies are aimed at determining the components that confer protection in our murine challenge system.

Consistent with the current view on protection from cytopathic viruses (9), studies of JE virus suggest that preexisting antibodies provide the critical and predictive factor in protection. In our previous experiments, recombinant vaccinia viruses that express the E protein synthesized in a misfolded form in infected cells failed to induce NEUT antibodies and provided little protection from challenge (25). In the present study, using DNA vaccines, we discovered an immunization strategy in which animals with low or undetectable levels of NEUT antibodies were protected from challenge. Following challenge, the sera from these animals contained high levels of NEUT antibodies, indicating a significant secondary immune response due to the challenge virus (probably due to replication at peripheral sites). We previously reported the replication of challenge virus in mice which had high prechallenge NEUT titers against JE virus (15), indicating that sterile immunity may be difficult to achieve in our challenge system.

Nevertheless, the data presented in this paper suggest that protection induced by delivery of JE virus gene subsets by recombinant viruses or as naked DNA may result from a different mechanism. Thus, in addition to its usefulness as a vaccine candidate, naked DNA immunization could be useful for elucidating the mechanisms of protection against flavivirus diseases.

## ACKNOWLEDGMENTS

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## REFERENCES

1. Allison, S. L., K. Stadler, C. W. Mandl, C. Kunz, and F. X. Heinz. 1995. Synthesis and secretion of recombinant tick-borne encephalitis virus protein E in soluble and particulate form. *J. Virol.* 69:5816-5820.
2. Cooney, E. L., A. C. Collier, P. D. Greenberg, R. W. Coombs, J. Zartling, D. E. Argitt, M. C. Hoffman, S. L. Hu, and L. Corey. 1991. Safety of and immunological response to a recombinant vaccinia virus vaccine expressing HIV envelope glycoprotein. *Lancet* 337:567-572.
3. Donnelly, J. J., J. B. Ulmer, and M. A. Liu. 1994. Immunization with DNA. *J. Immunol. Methods* 176:145-152.
4. Fonseca, B. A. L., S. Pincus, R. E. Shope, E. Paoletti, and P. W. Mason. 1994. Recombinant vaccinia viruses co-expressing dengue-1 glycoproteins prM and E induce neutralizing antibodies in mice. *Vaccine* 12:279-285.
5. Gagnon, S. J., W. Zeng, I. Kurane, and F. A. Ennis. 1996. Identification of two epitopes on the dengue 4 virus capsid protein recognized by a serotype-specific and a panel of serotype-cross-reactive human CD4<sup>+</sup> cytotoxic T-lymphocyte clones. *J. Virol.* 70:141-147.
6. Germain, R. N. 1993. Antigen processing and presentation, p. 629-676. *In* W. E. Paul (ed.), *Fundamental immunology*, 3rd ed. Raven Press Ltd., New York, N.Y.
7. Henchal, E. A., L. S. Henchal, and J. J. Schlesinger. 1988. Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. *J. Gen. Virol.* 69:2101-2107.
8. Johnson, M. P., C. A. Meitin, B. S. Bender, and P. A. Small, Jr. 1988. Passive immune serum inhibits antibody response to recombinant vaccinia virus, p. 189-192. *In* H. Ginsberg, F. Brown, R. A. Lerner, and R. M. Chanock (ed.), *Vaccines 88*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. Kagi, D., and H. Hengartner. 1996. Different roles for cytotoxic T cells in the control of infections with cytopathic versus noncytopathic viruses. *Curr. Opin. Immunol.* 8:472-477.
10. Kaufman, B. M., P. L. Summers, D. R. Dubois, W. H. Cohen, M. K. Gentry, R. L. Timchak, D. S. Burke, and K. H. Eckels. 1989. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. *Am. J. Trop. Med. Hyg.* 41:576-580.
11. Kimura-Kuroda, J., and K. Yasui. 1988. Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies. *J. Immunol.* 141:3606-3610.
12. Kochel, T., S. J. Wu, K. Raviprakash, P. Hobart, S. Hoffman, K. Porter, and C. Hayes. 1997. Inoculation of plasmids expressing the dengue-2 envelope gene elicits neutralizing antibodies in mice. *Vaccine* 15:547-552.
13. Konishi, E., I. Kurane, P. W. Mason, R. E. Shope, and F. A. Ennis. 1997. Poxvirus-based Japanese encephalitis vaccine candidates induce JE virus-specific CD8<sup>+</sup> cytotoxic T lymphocytes in mice. *Virology* 227:353-360.
14. Konishi, E., I. Kurane, P. W. Mason, R. E. Shope, N. Kanasa-Thanan, J. J. Smucny, C. H. Hoke, Jr., and F. A. Ennis. Induction of Japanese encephalitis virus-specific cytotoxic T lymphocytes in humans by poxvirus-based JE vaccine candidates. *Vaccine*, in press.
15. Konishi, E., S. Pincus, B. A. L. Fonseca, R. E. Shope, E. Paoletti, and P. W. Mason. 1991. Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. *Virology* 185:401-410.
16. Konishi, E., S. Pincus, E. Paoletti, W. W. Laegreid, R. E. Shope, and P. W. Mason. 1992. A highly attenuated host-range restricted vaccinia virus strain, NYVAC, encoding the prM, E and NS1 genes of Japanese encephalitis virus prevents JEV viremia in swine. *Virology* 190:454-458.
17. Konishi, E., S. Pincus, E. Paoletti, R. E. Shope, T. Burrage, and P. W. Mason. 1992. Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. *Virology* 188:714-720.
18. Konishi, E., S. Pincus, E. Paoletti, R. E. Shope, and P. W. Mason. 1994. Avipox virus-vectored Japanese encephalitis virus vaccines: use as vaccine candidates in combination with purified subunit immunogens. *Vaccine* 12: 633-638.



19. Kozak, M. 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 44: 283–292.
20. Kurane, I., Y. Okamoto, L. C. Dai, L. L. Zeng, M. A. Brinton, and F. A. Ennis. 1995. Flavivirus-cross-reactive, HLA-DR15-restricted epitope on NS3 recognized by human CD4<sup>+</sup> CD8<sup>+</sup> cytotoxic T lymphocyte clones. *J. Gen. Virol.* 76:2243–2249.
21. Livingston, P. G., I. Kurane, L. C. Dai, Y. Okamoto, C. J. Lai, R. Men, S. Karaki, M. Takiguchi, and F. A. Ennis. 1995. Dengue virus-specific, HLA-B35-restricted, human CD8<sup>+</sup> cytotoxic T lymphocyte (CTL) clones. Recognition of NS3 amino acids 500 to 508 by CTL clones of two different serotype specificities. *J. Immunol.* 154:1287–1295.
22. Manickan, E., K. L. Karem, and B. T. Rouse. 1997. DNA vaccines—a modern gimmick or a boon to vaccinology? *Crit. Rev. Immunol.* 17:139–154.
23. Mason, P. W. 1989. Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology* 169:354–364.
24. Mason, P. W., J. M. Dalrymple, M. K. Gentry, J. M. McCown, C. H. Hoke, Jr., D. S. Burke, M. J. Fournier, and T. L. Mason. 1989. Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. *J. Gen. Virol.* 70:2037–2049.
25. Mason, P. W., S. Pincus, M. J. Fournier, T. L. Mason, R. E. Shope, and E. Paoletti. 1991. Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. *Virology* 180:294–305.
26. McAda, P. C., P. W. Mason, C. S. Schmaljohn, J. M. Dalrymple, T. L. Mason, and M. J. Fournier. 1987. Partial nucleotide sequence of the Japanese encephalitis virus genome. *Virology* 158:348–360.
27. Murali-Krishna, K., V. Ravi, and R. Manjunath. 1996. Protection of adult but not newborn mice against lethal intracerebral challenge with Japanese encephalitis virus by adoptively transferred virus-specific cytotoxic T lymphocytes: requirement for L3T4<sup>+</sup> T cells. *J. Gen. Virol.* 77:705–714.
28. Phillips, R. J., K. Venugopal, and T. Brooks. 1996. Immunisation with DNA polynucleotides protects mice against lethal challenge with St. Louis encephalitis virus. *Arch. Virol.* 141:743–749.
29. Pincus, S., P. W. Mason, E. Konishi, B. A. L. Fonseca, R. E. Shope, C. M. Rice, and E. Paoletti. 1992. Recombinant vaccinia virus producing the prM and E proteins of yellow fever virus protects mice from lethal yellow fever encephalitis. *Virology* 187:290–297.
30. Pugachev, K. V., P. W. Mason, and T. K. Frey. 1995. Sindbis vectors suppress secretion of subviral particles of Japanese encephalitis virus from mammalian cells infected with SIN-JEV recombinants. *Virology* 209:155–166.
31. Robinson, H. L., L. A. Hunt, and R. G. Webster. 1993. Protection against a lethal influenza virus challenge by immunization with a haemagglutinin expressing plasmid DNA. *Vaccine* 11:957–960.
32. Rolph, M. S., and I. A. Ramshaw. 1997. Recombinant viruses as vaccines and immunological tools. *Curr. Opin. Immunol.* 9:517–524.
33. Rooney, J. F., C. Wohlenberg, K. J. Cremer, B. Moss, and A. L. Notkins. 1988. Immunization with a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: long-term protection and effect of revaccination. *J. Virol.* 62:1530–1534.
34. Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, and A. Friedman. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 259:1745–1749.
35. Wang, B., K. E. Ugen, V. Srikantan, M. G. Agadjanyan, K. Dang, Y. Refaeli, A. I. Sato, J. Boyer, W. V. Williams, and D. B. Weiner. 1993. Gene inoculation generates immune responses against human immunodeficiency virus type 1. *Proc. Natl. Acad. Sci. USA* 90:4156–4160.
36. Xiang, Z. Q., S. Spitalnik, M. Tran, W. H. Wunner, J. Cheng, and H. C. J. Ertl. 1994. Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. *Virology* 199:132–140.

## Dengue Virus-Specific Human CD4<sup>+</sup> T-Lymphocyte Responses in a Recipient of an Experimental Live-Attenuated Dengue Virus Type 1 Vaccine: Bulk Culture Proliferation, Clonal Analysis, and Precursor Frequency Determination

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We analyzed the CD4<sup>+</sup> T-lymphocyte responses to dengue, West Nile, and yellow fever viruses 4 months after immunization of a volunteer with an experimental live-attenuated dengue virus type 1 vaccine (DEN-1 45A25). We examined bulk culture proliferation to noninfectious antigens, determined the precursor frequency of specific CD4<sup>+</sup> T cells by limiting dilution, and established and analyzed CD4<sup>+</sup> T-cell clones. Bulk culture proliferation was predominantly dengue virus type 1 specific with a lesser degree of cross-reactive responses to other dengue virus serotypes, West Nile virus, and yellow fever virus. Precursor frequency determination by limiting dilution in the presence of noninfectious dengue virus antigens revealed a frequency of antigen-reactive cells of 1 in 1,686 peripheral blood mononuclear cells (PBMC) for dengue virus type 1, 1 in 9,870 PBMC for dengue virus type 3, 1 in 14,053 PBMC for dengue virus type 2, and 1 in 17,690 PBMC for dengue virus type 4. Seventeen CD4<sup>+</sup> T-cell clones were then established by using infectious dengue virus type 1 as antigen. Two patterns of dengue virus specificity were found in these clones. Thirteen clones were dengue virus type 1 specific, and four clones recognized both dengue virus types 1 and 3. Analysis of human leukocyte antigen (HLA) restriction revealed that five clones are HLA-DRw52 restricted, one clone is HLA-DP3 restricted, and one clone is HLA-DP4 restricted. These results indicate that in this individual, the CD4<sup>+</sup> T-lymphocyte responses to immunization with live-attenuated dengue virus type 1 vaccine are predominantly serotype specific and suggest that a multivalent vaccine may be necessary to elicit strong serotype-cross-reactive CD4<sup>+</sup> T-lymphocyte responses in such individuals.

Dengue viruses are members of the family *Flaviviridae*; they are transmitted by *Aedes aegypti* mosquitoes. There are an estimated 100 million cases of dengue virus infection in the world each year, occurring predominantly in the tropical and subtropical regions of Central and South America and Southeast Asia (5). The majority of infections are asymptomatic or cause a self-limited illness known as dengue fever. The more severe form of dengue infection, dengue hemorrhagic fever-dengue shock syndrome, is characterized by plasma leakage and may be life threatening (5). Because of the significant morbidity and mortality attributable to dengue viruses, efforts are under way to develop a safe and immunogenic vaccine.

There are four serotypes of dengue virus, types 1, 2, 3, and 4. Immunity to the infecting serotype is believed to be lifelong (5, 6). Following natural infection, cross-reactive antibody responses are elicited; however, protection to heterologous dengue virus serotypes is short lived, on the order of several months (14). Cross-reactive T-cell responses have been observed in humans following natural infection or immunization with live-attenuated dengue virus vaccines (4, 10). Our laboratory has demonstrated dengue virus serotype cross-reactive CD4<sup>+</sup> lymphocyte responses in the bulk culture of the peripheral blood mononuclear cells (PBMC) of an individual immunized against dengue virus type 3 (11). Six

patterns of dengue virus and flavivirus specificities were noted in CD4<sup>+</sup> T-cell clones from this individual: dengue virus type 3 serotype specific, dengue virus subcomplex specific (dengue virus types 1, 2, and 3, and dengue virus types 2, 3, and 4), dengue virus serotype cross-reactive, and two patterns of flavivirus cross-reactivity (8). Cell-mediated immunity is believed to be necessary for the control of virus infections. The level of T-cell recognition of viruses has been studied to a limited degree in the evaluation of the immunogenicity of experimental human immunodeficiency virus vaccines (13). In our study, T-cell memory responses induced in a dengue virus-naïve recipient by an experimental live-attenuated dengue virus type 1 vaccine were analyzed at bulk culture, precursor frequency, and clonal levels to determine the immunogenicity of this vaccine. These studies reveal a high CD4<sup>+</sup> T-cell precursor frequency to dengue virus type 1 (1 in 1,686) compared with the other three serotypes. Of 17 cytotoxic CD4<sup>+</sup> clones, 13 were dengue virus type 1 specific and 4 were dengue virus type 1-dengue virus type 3 cross-reactive.

### MATERIALS AND METHODS

**Viruses.** Dengue virus type 1 (Hawaii strain) and dengue virus type 2 (New Guinea C strain) were provided by Walter E. Brandt, Walter Reed Army Institute of Research. Dengue virus type 3 (CH53489 strain), was provided by Bruce L. Innis, Armed Forces Institute of Medical Science, Bangkok,

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Thailand. Dengue virus type 4 (814669 strain) was provided by Jack McCown, Walter Reed Army Institute of Research. Yellow fever virus (YFV) (17D strain) was provided by Jacob J. Schlesinger, University of Rochester School of Medicine and Dentistry. West Nile virus (WNV) (E101 strain) was provided by Margo Brinton, Georgia State University. Viruses were propagated in C6/36 mosquito cells to titers of  $10^7$  to  $10^8$  PFU/ml as previously described (9) and frozen at  $-70^\circ\text{C}$  until use.

**Experimental dengue virus type 1 vaccine (DEN-1 45AZ5).** The virus seed was originally isolated by Leon Rosen, National Institute of Allergy and Infectious Diseases, from the serum of a child with dengue fever. The virus was isolated and subsequently passaged in diploid fetal rhesus lung (FRh1-2) cell culture. A small plaque was selected and mutagenized with 5-azacytidine. This seed was subsequently prepared by the Salk Institute at the Swiftwater, Pa., facility. The vaccine was administered to two healthy volunteers, who subsequently developed classic dengue fever (12). To further attenuate the strain, the virus was passaged 27 times in primary dog kidney cells (PDK) and vaccines were prepared in FRh1-2 cells from PDK passages 10, 20, and 27. The PDK passage 27 vaccine was given to 10 individuals; only 60% developed neutralizing antibodies (unpublished data).

**Preparation of dengue virus antigens.** Dengue virus antigens, YFV antigen, and WNV antigen were prepared as previously described (10). Briefly, Vero cells were infected with viruses at a multiplicity of infection of 1 PFU per cell and cultured in minimal essential medium with 2% fetal calf serum (FCS). Cells were removed with cell scrapers when 50% cytopathic effect was observed. They were washed three times in phosphate-buffered saline (PBS) at  $4^\circ\text{C}$ , treated with 0.025% glutaraldehyde (Sigma Chemical Co., St. Louis, Mo.) in PBS for 15 min at  $4^\circ\text{C}$ , washed three times with PBS, and resuspended in RPMI 1640 medium. Cells were sonicated and centrifuged at  $1,500 \times g$  for 10 min. The supernatant fluid was collected and used as the virus antigen. Control antigen was prepared with uninfected Vero cells in similar fashion.

**Human PBMC.** Peripheral blood specimens were obtained from the donor 4 months after vaccination with DEN-1 45AZ5 PDK passage 27. This donor was a 38-year-old white man who had never traveled outside of the United States. His serum did not contain antibody to dengue viruses prior to enrollment in a phase I clinical trial at University of Maryland Center for Vaccine Development. Following immunization, this volunteer experienced a mild dengue illness between days 10 and 19, with rash, malaise, arthralgia, myalgia, eye pain, headache, and nausea but no fever. He did not have detectable viremia but developed immunoglobulin M antibody to dengue virus type 1 and a neutralizing antibody titer of 1:90 at 31 days postvaccination. PBMC were separated by density gradient centrifugation with Ficoll-Hypaque (2). The cells were resuspended at  $10^7/\text{ml}$  in RPMI medium with 10% FCS (Sigma) and 10% dimethyl sulfoxide and cryopreserved until use.

**Proliferative responses of PBMC.** Proliferation assays of PBMC were performed as previously described (10). PBMC ( $1.5 \times 10^5$  to  $2.5 \times 10^5$ ) were cultured with viral antigens at various dilutions in 0.2 ml of AIM-V medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% human AB serum (Advanced Biotechnologies, Inc., Columbia, Md.) in 96-well round-bottom microtiter plates (Costar, Cambridge, Mass.) at  $37^\circ\text{C}$  for 6 days. The cells were pulsed with  $1.25 \mu\text{Ci}$  of tritiated thymidine ( $[^3\text{H}]\text{TdR}$ ) for 6 h before

harvest with a multiharvester (Titertek; Skatron Inc. Sterling, Va.).  $[^3\text{H}]\text{TdR}$  incorporation was counted in a liquid scintillation counter (1205 Betaplate; Pharmacia, Wallac Oy, Finland).

**Dengue virus-specific precursor frequency by the limiting-dilution method.** The limiting-dilution assay was based on the method of Van Oers et al. (17). Thirty replicate wells containing various numbers (range, 625 to 20,000 cells per well) of PBMC were cultured in 0.2 ml of AIM-V containing 10% human AB serum with 1:320 diluted dengue virus antigen, control antigen, or no antigen in the presence of  $7 \times 10^4$  autologous  $\gamma$ -irradiated (3,500 rads) PBMC and 2 U of recombinant human interleukin-2 (Collaborative Research, Inc., Bedford, Mass.) per ml in 96-well round-bottom microtiter plates. For cord blood PBMC, 18 replicate wells containing various numbers (range, 5,000 to 20,000 cells per well) of PBMC were prepared as above. After 6 days, 50  $\mu\text{l}$  of fresh medium containing 5 U of recombinant human interleukin-2 per ml was added to all wells. The cultures were pulsed 4 to 6 days later with  $1.25 \mu\text{Ci}$  of  $[^3\text{H}]\text{TdR}$  per well for 8 h and harvested as above. Positive cultures were defined as having levels of  $[^3\text{H}]\text{TdR}$  incorporation that were greater than 3 standard deviations above the mean of the 30 replicate unstimulated wells. By this method, the false-positivity rate is limited to less than 0.14% (3).

**Establishment of dengue virus-specific T-cell clones by limiting dilution.** Dengue virus-specific T-cell clones were established as previously reported (11). PBMC ( $4 \times 10^6$  cells) were cultured with 1:2-diluted dengue virus type 1 in RPMI 1640 medium containing 10% heat-inactivated pooled human AB serum, penicillin-streptomycin, glutamine, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Sigma) and placed in 96-well round-bottom microtiter plates. The plates were incubated for 7 days at  $37^\circ\text{C}$ . On day 7, the cells were collected and cultured at a concentration of 1, 3, 10, and 100 cells per well in 0.15 ml of AIM-V containing 10% pooled human AB serum, 10% T-cell growth factor (Cellular Products, Inc., Buffalo, N.Y.),  $10^5$   $\gamma$ -irradiated (3,500 rads) autologous PBMC, and 1:3-diluted dengue virus type 1. On day 4, 0.05 ml of fresh medium with human AB serum and T-cell growth factor was added. On day 7, 0.1 ml of supernatant was removed and fresh medium was added as above. In addition,  $10^5$   $\gamma$ -irradiated (3,500 rads) autologous PBMC and 1:2-diluted dengue virus type 1 were added. On days 14 and 21, growing cells were transferred to 48-well flat-bottom plates (Costar) and cultured with  $10^6$   $\gamma$ -irradiated PBMC.

**Establishment of lymphoblastoid cell lines.** PBMC ( $2 \times 10^6$  cells) were cultured in RPMI 1640 medium containing 10% FCS, penicillin, streptomycin, glutamine, and HEPES in the presence of 1:3-diluted Epstein-Barr virus from an infected marmoset cell line, B95-8 (ATCC), in 24-well flat-bottom plates (Costar) (15).

**Preparation of target cells.** Lymphoblastoid cells ( $4 \times 10^5$  cells) were cultured for 16 to 20 h in RPMI 1640 medium containing 10% FCS and 1:50 diluted dengue virus, YFV, WNV, or control antigens. The cells were then washed and labeled with  $^{51}\text{Cr}$  (Dupont NEN, Boston, Mass.) to be used as target cells.

**Cytotoxicity assays.** Target cells ( $4 \times 10^5$  cells) were labeled with  $0.25 \text{ mCi}$  of  $^{51}\text{Cr}$  in 0.2 ml RPMI medium plus 10% FCS for 60 min, washed three times with RPMI medium plus 10% FCS, and resuspended at  $10^6$  cells per ml. Targets were plated at  $10^3$  per well in 0.1 ml RPMI medium plus 10% FCS in V-bottom microtiter plates (Costar). Effector cells were added at different concentrations in triplicate in 0.1 ml

TABLE 1. Proliferation responses of the PBMC of a dengue virus type 1 vaccine recipient to dengue and flavivirus antigens in bulk culture<sup>a</sup>

Antigen	<sup>3</sup> H]TdR incorporation (cpm) at viral antigen dilution of:		
	1:160	1:320	1:640
Dengue virus type 1	41,001	54,265	34,832
Dengue virus type 2	16,387	16,512	7,322
Dengue virus type 3	16,412	17,631	8,030
Dengue virus type 4	7,220	6,501	4,417
WNV	894	13,262	9,043
YFV	14,649	7,642	5,539
Control	161	1,586	1,856

<sup>a</sup> PBMC ( $2 \times 10^5$  cells) were incubated for 6 days in the presence of serial dilutions of dengue virus, flavivirus, and control antigens. Cells were pulsed with  $1.25 \mu\text{Ci}$  of <sup>3</sup>H]TdR for 6 h, and <sup>3</sup>H]TdR incorporation was measured. No antigen control mean is 1,379 cpm.

of RPMI medium plus 10% FCS. The plates were centrifuged at  $200 \times g$  for 5 min and then incubated at  $37^\circ\text{C}$  for 5 h. Following incubation, the supernatant fluids were harvested and counted in an automatic gamma counter. The percent specific <sup>51</sup>Cr release was calculated from the following formula:  $[(\text{cpm experimental release} - \text{cpm spontaneous release}) / (\text{cpm maximal release} - \text{cpm spontaneous release})] \times 100$ .

**Inhibition of lysis of dengue virus type 1 antigen-pulsed target cells by monoclonal antibodies directed against class II human leukocyte antigens (HLA).** Monoclonal antibodies B7/21.7, S3/4, and OKIa1 recognize HLA-DP, HLA-DQ, and HLA-DR determinants, respectively. B7/21.7 and S3/4 were kindly provided by Nancy Reinsmoen, University of Minnesota, Minneapolis. OKIa1 was purchased from Ortho Diagnostic Systems, Inc., Raritan, N.J. The 10th International Histocompatibility Workshop lymphoblastoid cell lines (American Society for Histocompatibility and Immunogenetics, Lenexa, Kans.) were used as target cells. A total of  $10^3$  <sup>51</sup>Cr-labeled target cells in 0.05 ml of RPMI medium plus 10% FCS were incubated with 0.05 ml of 1:20-diluted monoclonal antibodies for 30 min. The effector cells were then added in 0.1 ml, and the mixture was incubated for 6 h. The percent specific <sup>51</sup>Cr release was determined as described above.

**Phenotype analysis.** Cell surface antigens CD3, CD4 and CD8 were analyzed by using fluorescein isothiocyanate-conjugated monoclonal antibodies anti-Leu4, anti-Leu2 and anti-Leu3 (Becton Dickinson Co., Mountain View, Calif.), respectively. Briefly,  $20 \mu\text{l}$  of antibody was added to  $0.5 \times 10^6$  to  $1 \times 10^6$  cells and incubated for 30 min at  $4^\circ\text{C}$ . The cells were then washed twice in cold PBS and analyzed by a fluorescence-activated cell sorter or by fluorescence microscopy.

**Statistical analysis.** Antigen-reactive cell frequencies were calculated by using a computer-assisted Taswell analysis provided by Richard A. Miller, University of Michigan, Ann Arbor (16).

## RESULTS

**Proliferative responses of PBMC to noninfectious dengue virus antigens.** We first examined the dengue virus serotype specificity and flavivirus specificity of CD4<sup>+</sup> memory T cells in bulk culture proliferation assays. PBMC were cultured with antigens of the four dengue virus serotypes, WNV,

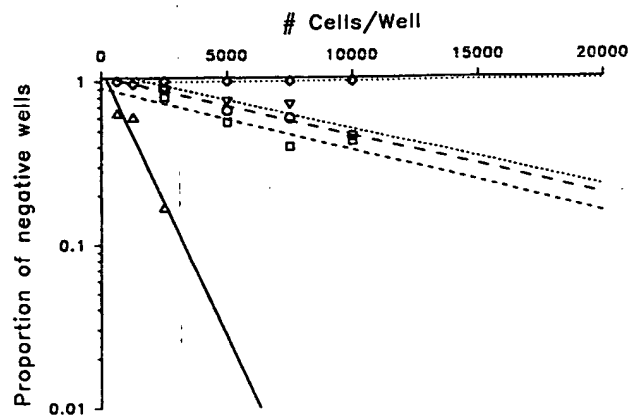


FIG. 1. Precursor frequency analysis of T lymphocytes specific for dengue virus type 1, 2, 3 and 4 antigens. Thirty replicate wells containing various numbers (625 to 10,000 cells per well) of PBMC were cultured in 0.2 ml of AIM-V containing 10% human AB serum with 1:320 diluted dengue virus antigen, control antigen, or no antigen in the presence of  $7 \times 10^4$  autologous  $\gamma$ -irradiated PBMC and 2 U of recombinant interleukin-2 per ml. A  $50\text{-}\mu\text{l}$  volume of media containing 5 U of interleukin-2 per ml was added to all the wells on day 6. <sup>3</sup>H]TdR incorporation was examined on day 10. Positive cultures were defined as having levels of <sup>3</sup>H]TdR incorporation that were greater than 3 standard deviations above the mean of 30 replicate unstimulated wells. Symbols:  $\diamond$ , control antigen;  $\Delta$ , dengue virus type 1 antigen;  $\circ$ , dengue virus type 2 antigen;  $\square$ , dengue virus type 3 antigen;  $\nabla$ , dengue virus type 4 antigen.

YFV, or control antigens at 1:160, 1:320 and 1:640 dilutions, and the levels of <sup>3</sup>H]TdR incorporation were assessed (Table 1). Dengue virus type 1 antigen induced the highest level of proliferation, and dengue virus types 2, 3, and 4, WNV, and YFV antigens induced proliferation to a lesser extent. Control antigen did not induce a proliferative response. T lymphocytes that respond to these noninfectious dengue virus antigens are predominantly CD4<sup>+</sup> (10, 11, 18). These results suggest that dengue virus-specific CD4<sup>+</sup> memory T lymphocytes of this donor are predominantly dengue virus type 1 specific and that dengue virus- and flavivirus-cross-reactive CD4<sup>+</sup> memory T lymphocytes are present to a lesser degree.

**Precursor frequency of memory CD4<sup>+</sup> T cells to dengue viruses.** To quantitate the level of CD4<sup>+</sup> memory T cells induced in this donor by the dengue virus type 1 vaccine, we performed a precursor frequency analysis by limiting dilution in the presence of noninfectious dengue virus antigens as described in Materials and Methods. Results of a representative experiment are shown in Fig. 1. The CD4<sup>+</sup> T-cell precursor frequency is highest for dengue virus type 1 (1 in 1,686 PBMC [95% confidence interval, 1,297 to 2,410]) and is followed by those for dengue virus type 3 (1 in 9,870 PBMC [95% confidence interval, 7,781 to 13,492]), dengue virus type 2 (1 in 14,053 PBMC [95% confidence interval, 10,718 to 20,400]), and dengue virus type 4 (1 in 17,690 PBMC [95% confidence interval, 13,204 to 26,792]). As a negative control in a parallel experiment, cord blood PBMC cultured in similar fashion showed no antigen-reactive cells when 18 replicate wells were plated at up to 20,000 PBMC per well (data not shown). These data confirm the results with the bulk cultures, which suggest that the CD4<sup>+</sup> memory T lymphocyte induced by the DEN-1 45A25 vaccine is predominantly dengue virus type 1 specific.

**Virus and serotype specificity of responding cells by estab-**

TABLE 2. Dengue serotype and flavivirus specificities of CD4<sup>+</sup> cytotoxic T-cell clones<sup>a</sup>

Clone	E:T <sup>b</sup>	% Specific <sup>51</sup> Cr release <sup>c</sup> by:							NoAg
		D1Ag <sup>d</sup>	D2Ag	D3Ag	D4Ag	WNV <sup>d</sup>	YFV <sup>d</sup>	Cont	
1	16:1	27	0	0	0	0	0	0	0
3	15:1	30	0	0	0	0	0	0	0
5	12:1	80	0	0	0	0	0	0	0
6	3:1	50	0	0	0	0	0	0	0
7	14:1	74	0	0	0	0	0	0	0
9	14:1	44	0	0	0	0	0	0	0
10	13:1	87	0	28	0	0	0	0	0
13	6:1	44	0	0	0	0	0	0	0
14	16:1	63	0	0	0	0	0	0	0
15	6:1	71	0	64	0	0	0	0	0
16	9:1	27	0	30	0	0	0	0	0
17	6:1	59	0	0	0	0	0	0	0
18	9:1	75	0	66	0	0	0	0	0
20	7:1	70	0	0	0	0	0	0	0
24	15:1	75	0	0	0	0	0	0	0
32	13:1	54	0	0	0	0	0	0	0
33	5:1	22	0	0	0	0	0	0	0

<sup>a</sup> Target cells ( $1.0 \times 10^3$ ) were incubated with effector cells for 5 h. <sup>51</sup>Cr release was calculated as described in Materials and Methods.

<sup>b</sup> E:T, effector-to-target cell ratio.

<sup>c</sup> Underlines indicate significant levels of lysis. Cont, control antigen; NoAg, no antigen.

<sup>d</sup> Dengue virus antigens, WNV antigen, and YFV antigen were prepared as described in Materials and Methods.

lishment of dengue virus-specific CD4<sup>+</sup> T-cell clones. To further analyze the dengue virus serotype specificity and flavivirus specificity of CD4<sup>+</sup> memory T cells in this donor, we established dengue virus-specific CD4<sup>+</sup> CD8<sup>-</sup> T-cell clones by limiting dilution as described in Materials and Methods. Seventeen CD4<sup>+</sup> T-cell clones were established. The flavivirus specificities and dengue virus serotype specificities of the clones were examined in cytotoxic T-lymphocyte assays. The results of these assays are shown in Table 2. Thirteen clones were dengue virus type 1 specific. Four clones recognized both dengue virus type 1 and 3 antigens. Dengue virus types 2 and 4, WNV, and YFV were not recognized by any of these CD4<sup>+</sup> T-cell clones. These results are consistent with those obtained in bulk culture proliferation assays and suggest that dengue virus type 1-specific CD4<sup>+</sup> T cells were induced predominantly by immunization with this experimental dengue virus type 1 vaccine.

**HLA restriction of the lysis of target cells by CD4<sup>+</sup> T-cell clones.** HLA restriction of the lysis of target cells by six dengue virus-specific CD4<sup>+</sup> T-cell clones was first examined by using monoclonal antibodies to HLA class II molecules (Table 3). Monoclonal antibody to HLA-DP inhibited the lysis of target cells by dengue virus type 1-specific clones 17 and 20. Monoclonal antibody to HLA-DQ did not inhibit the lysis of target cells. Monoclonal antibody to HLA-DR inhibited the lysis of dengue virus type 1-specific clones 7, 9, and 24 and dengue virus type 1-dengue virus type 3 cross-reactive clone 10.

To further delineate the HLA restriction of these dengue-specific CD4<sup>+</sup> T-cell clones, we analyzed the lysis of dengue virus type 1 antigen-pulsed allogeneic target cells (Table 4). Although the HLA-DP type of the donor is not known, we can infer from the antibody-blocking pattern and from allo-target lysis that the dengue virus type 1-specific clone 17 is HLA-DP4 restricted and that dengue virus type 1-specific

TABLE 3. Inhibition of target cell lysis by monoclonal antibodies directed against class II HLA<sup>a</sup>

Clone	% Specific <sup>51</sup> Cr release <sup>b</sup> by:				No Ag
	Dengue virus type 1 antigen				
	No antibody	Anti-DP	Anti-DQ	Anti-DR	
7	92	88	81	46 <sup>c</sup>	1
9	74	71	67	53	2
10	93	88	84	67	2
17	41	4	4 <sup>c</sup>	18	4
20	81	42	97	71	5
24	52	58	63	12	0

<sup>a</sup> A total of  $10^3$  target cells were incubated with effector cells for 6 h in the presence of monoclonal antibodies at final dilution of 1:80. B7/21.7, S3/4, and OK1a1 were used as anti-HLA-DP, anti-HLA-DQ, and anti-HLA-DR, respectively.

<sup>b</sup> The effector-to-target cell ratio was 8:1 for clone 17; 18:1 for clone 10; 19:1 for clones 7 and 20; and 20:1 for clones 9 and 24.

<sup>c</sup> Underlines indicate significant inhibition of lysis.

clone 20 is HLA-DP3 restricted. Dengue virus type 1-specific clones 7, 9, and 24 and dengue virus type 1-dengue virus type 3 cross-reactive clone 10 appear to be HLA-DRw52 restricted.

## DISCUSSION

In this study, we analyzed the dengue virus-specific CD4<sup>+</sup> memory T cells in a volunteer who had received an experimental live-attenuated dengue virus type 1 vaccine 4 months earlier. Proliferation to noninfectious dengue virus antigens in bulk culture was predominantly dengue virus type 1 specific, and there was a lower level of serotype-cross-reactive responses. Similar bulk culture responses were noted in another donor whose lymphocytes were examined following natural infection with dengue virus type 1 (11). We also determined the CD4<sup>+</sup> precursor frequency and analyzed the responses of cytotoxic CD4<sup>+</sup> CD8<sup>-</sup> T cells at the clonal level. The CD4<sup>+</sup> T-cell precursor frequency was highest for dengue virus type 1 and lower for the other dengue virus serotypes. Cord blood PBMC did not react to dengue virus antigens. Of 17 CD4<sup>+</sup> T-cell clones established from this donor, 13 were dengue virus type 1 specific and 4 were dengue virus type 1 and dengue virus type 3 cross-reactive. Six clones were analyzed for HLA restriction patterns. One clone was HLA-DP3 restricted, and one was HLA-DP4 restricted. Four clones were HLA-DRw52 restricted. These clones did not lyse all of the HLA-DRw52-matched allogeneic target cells, but this may be due to different subtypes of HLA-DRw52.

These results differ from our previous results, which were obtained by analyzing PBMC of individuals who were infected with dengue virus type 3 or 4 (references 8 and 11 and unpublished observation). In those individuals, bulk culture and clonal analysis revealed more serotype and subcomplex cross-reactivity than was observed in our dengue virus type 1-immune donor. The reasons for this difference are not clear; however, possible explanations are as follows. (i) The differences in the levels of serotype-cross-reactive CD4<sup>+</sup> memory T cells after primary infection may be due to the differences of HLA haplotype. T cells of individuals with certain HLA types may dominantly recognize serotype-specific T-cell epitopes, whereas others with different HLA types recognize serotype-cross-reactive epitopes. (ii) Dengue virus type 1 may produce a more serotype-specific CD4<sup>+</sup>

TABLE 4. Determination of HLA class II restriction of CD4<sup>+</sup> T-cell clones with dengue virus type 1 antigen-pulsed allogeneic target cells<sup>a</sup>

Target	HLA class II type <sup>b</sup>				% Specific <sup>51</sup> Cr release <sup>c</sup>					
	DR	DR	DP	DQ	7	9	10	17	20	24
<b>Expt 1</b>										
Autologous	1/w6	w52	— <sup>d</sup>	w1	95	85	97	ND <sup>e</sup>	87	83
9004	1	—	4	w5 (w1)	0	2	81	ND	0	0
9011	w15	—	2/4	w6 (w1)	0	0	0	ND	0	0
9038	w12	w52	2	w7	65	32	44	ND	0	0
9049	7	w52	1	w2	0	0	11	ND	0	0
3099	1	—	—	w1	0	2	33	ND	0	0
<b>Expt 2</b>										
Autologous	1/w6	w52	—	w1	100	73	93	78	87	81
9022	w17	w52	3	w2	10	8	3	6	38	29
9052	7	w53	4	w9	0	0	0	32	0	0
9074	9	w53	2/5	w9	0	0	10	5	0	1
9087	3	w52	3/4	w2	3	0	5	25	11	0
3153	w6	—	—	—	6	0	3	9	0	1
JK	2	—	2	w1	0	2	0	0	0	32
<b>Expt 3</b>										
Autologous	1/w6	w52	—	w1	ND	31	ND	15	ND	6
9062	w13	w52	4	w6 (w1)	ND	50	ND	0	ND	0
GM-11	w6	—	—	—	ND	57	ND	0	ND	0
<b>Expt 4</b>										
Autologous	1/w6	w52	—	w1	25	22	38	11	38	19
3103	4	—	2	—	0	0	0	1	0	0
3106	5	—	2	w1	26	10	13	1	0	0
CB	7	w53	4	w2	0	0	0	15	0	0
CP	5/7	w52/w53	4	w2/w3	31	23	14	13	0	0
PG	3/5	w52	—	w2/w3	54	20	39	22	10	0

<sup>a</sup> A total of  $1 \times 10^3$  targets were incubated with effectors for 5.5 h.<sup>b</sup> Known HLA loci which match donor HLA are underlined.<sup>c</sup> The effector-to-target cell ratio was 4:1 for clone 7 (experiments 2 and 4); 5:1 for clone 24 (experiment 3); 8:1 for clones 17 and 20 (experiment 4); 9:1 for clone 9 (experiment 3); 10:1 for clones 10 and 24 (experiment 2); 11:1 for clone 10 (experiment 4); 12:1 for clone 9 (experiment 1); 14:1 for clones 17 (experiment 3), 9 (experiment 4), and 24 (experiment 1); 15:1 for clone 17 (experiment 2); 16:1 for clones 9 and 20 (experiment 2); 17:1 for clone 24 (experiment 4); 19:1 for clone 7 (experiment 1); and 20:1 for clones 10 and 20 (experiment 1). Underlines indicate significant levels of lysis.<sup>d</sup> —, locus for which HLA type is not known.<sup>e</sup> ND, not done.

T lymphocyte response than the other serotypes of dengue virus because of the characteristic amino acid sequences of possible CD4<sup>+</sup> T-cell epitopes.

Although we used live dengue virus type 1 as the antigen to establish T-cell clones from this donor, only CD4<sup>+</sup> T-cell clones were isolated. We have successfully established HLA class I-restricted CD8<sup>+</sup> T-cell clones from the PBMC of a dengue virus type 4-immune donor as well as CD4<sup>+</sup> T-cell clones with infectious dengue virus as the antigen (unpublished observation). The dengue virus type 1 we used, however, does not infect monocytes-macrophages well compared with the other serotypes of dengue virus. It is possible that a virus that does not infect these cells is better processed as exogenous antigens and stimulates an HLA class II-restricted CD4<sup>+</sup> T lymphocyte response and hence production of CD4<sup>+</sup> T-cell clones.

The clonal analysis revealed that a majority of the CD4<sup>+</sup> T-cell clones were dengue virus type 1 serotype specific, although clonal analysis may not necessarily represent numerically the specificities of the T-cell population in an individual. A number of investigators have studied the virus-specific cytotoxic CD8<sup>+</sup> T-lymphocyte precursor frequency (1, 7); however, the CD4<sup>+</sup> T-cell precursor frequency has not been well characterized. We performed a CD4<sup>+</sup> precursor frequency determination for each dengue

virus serotype. The CD4<sup>+</sup> T-cell precursor frequency for dengue virus type 1 was quite high (1 in 1,686 PBMC) and was 5 to 10 times greater than the frequency for the other dengue serotypes. Analysis of the CD4<sup>+</sup> T-cell clones established from this individual reflects this ratio. None of the clones that we established recognized dengue virus type 2 or 4. According to the precursor frequencies for each serotype of virus, the number of CD4<sup>+</sup> T-cell clones specific for dengue virus types 2 and 4 would be expected to be 1 or 2 of the 17 clones. It is possible that the T-cell clones cross-reactive for dengue virus types 2 and 4 would have been apparent if a larger number of clones had been established. Another explanation is that serotype-cross-reactive CD4<sup>+</sup> T cells respond to dengue virus type 2, 3, or 4 to a higher degree than to dengue virus type 1. Therefore, although these serotype-cross-reactive CD4<sup>+</sup> T cells were detectable at low precursor frequencies by using antigens of other serotypes, they were not established as clones when dengue virus type 1 was used as the stimulating antigen.

The level of the dengue virus type 1-specific memory T cell in this study was high. This may be because the PBMC were obtained from this subject only 4 months after immunization. It will be important to monitor the changes in the levels of memory CD4<sup>+</sup> T cells in this subject. We also plan to determine the levels of dengue virus-specific CD8<sup>+</sup> T cells

in this individual. Although the relationship between the frequencies of virus-specific memory T cells and the ability to recover from infection is not known, the analysis of the precursor frequencies may provide useful information that could be used to understand the immunogenicity of experimental vaccines and help guide the timing of vaccination in future immunization strategies. The results in this paper suggest that in some individuals, the CD4<sup>+</sup> T-cell responses to primary dengue virus type 1 infection are predominantly serotype specific. Thus, if one is trying to induce strong cross-reactive memory T cells in such individuals by immunization, a multivalent dengue virus vaccine should be considered. It is important to determine the percentage of individuals who develop predominantly serotype-specific responses and whether there are correlations between HLA types and the predominant serotype-specific responses. These studies should provide useful information for the development of safe and effective dengue virus vaccines.

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#### REFERENCES

1. Borysiewicz, L. K., S. Graham, J. K. Hickling, P. D. Mason, and J. G. P. Sissons. 1988. Human cytomegalovirus-specific cytotoxic T cells: their precursor frequency and stage specificity. *Eur. J. Immunol.* 18:269-275.
2. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21(Suppl. 97):77-89.
3. Brett, S. J., A. E. Kingston, and M. J. Colston. 1987. Limiting dilution analysis of the human T cell response to mycobacterial antigens from BCG vaccinated individuals and leprosy patients. *Clin. Exp. Immunol.* 68:510-520.
4. Bukowski, J. F., I. Kurane, C. J. Lai, M. Bray, B. Falgout, and F. A. Ennis. 1989. Dengue virus-specific cross-reactive CD8<sup>+</sup> human cytotoxic T lymphocytes. *J. Virol.* 63:5086-5091.
5. Halstead, S. B. 1989. Pathogenesis of dengue: challenges to molecular biology. *Science* 239:476-481.
6. Hayes, E. B., and D. J. Gubler. 1992. Dengue and dengue hemorrhagic fever. *Pediatr. Infect. Dis. J.* 11:311-317.
7. Hoffenbach, A., P. Langlade-Demoyen, G. Dadaglio, E. Vilmer, F. Michel, C. Mayaud, B. Aufran, and F. Plata. 1989. Unusually high frequencies of HIV-specific cytotoxic T lymphocytes in humans. *J. Immunol.* 142:452-462.
8. Kurane, I., M. A. Brinton, A. L. Samson, and F. A. Ennis. 1991. Dengue virus-specific, human CD4<sup>+</sup> CD8<sup>+</sup> cytotoxic T-cell clones: multiple patterns of virus cross-reactivity recognized by NS3-specific T-cell clones. *J. Virol.* 65:1823-1828.
9. Kurane, I., D. Hebblewhite, W. E. Brandt, and F. A. Ennis. 1984. Lysis of dengue virus-infected cells by natural cell-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity. *J. Virol.* 52:223-229.
10. Kurane, I., B. L. Innis, A. Nisalak, C. Hoke, S. Nimmannitya, A. Meager, and F. A. Ennis. 1989. Human responses to dengue virus antigens. Proliferative responses and interferon gamma production. *J. Clin. Invest.* 83:506-513.
11. Kurane, I., A. Meager, and F. A. Ennis. 1989. Dengue virus-specific human T cell clones. Serotype crossreactive proliferation, interferon gamma production, and cytotoxic activity. *J. Exp. Med.* 170:763-775.
12. McKee, K. T., Jr., W. H. Bancroft, K. H. Eckels, R. R. Redfield, P. L. Summers, and P. K. Russell. 1987. Lack of attenuation of a candidate dengue 1 vaccine (45A25) in human volunteers. *Am. J. Trop. Med. Hyg.* 36:435-442.
13. Redfield, R. R., D. L. Birx, N. Ketter, E. Tramont, V. Polonis, C. Davis, J. F. Brundage, G. Smith, S. Johnson, A. Fowler, T. Wierzb, A. Shafferman, F. Volvovitz, C. Oster, D. Burke, and the Military Medical Consortium for Applied Retroviral Research. 1991. A phase I evaluation of the safety and immunogenicity of vaccination with recombinant gp160 in patients with early human immunodeficiency virus infection. *N. Engl. J. Med.* 324:1677-1684.
14. Sabin, A. B. 1952. Research on dengue during World War II. *Am. J. Trop. Med. Hyg.* 1:30-50.
15. Sly, W. S., G. S. Sekhon, R. Kennett, W. F. Bodmer, and J. Bodmer. 1976. Permanent lymphoid lines from genetically marked lymphocytes: success with lymphocytes recovered from frozen storage. *Tissue Antigens* 7:165-172.
16. Taswell, C. 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. *J. Immunol.* 126:1614-1619.
17. Van Oers, M. H. J., J. Pinkster, and W. P. Zeijlmeier. 1978. Quantification of antigen-reactive cells among human T lymphocytes. *Eur. J. Immunol.* 8:477-484.
18. Zivny, J., I. Kurane, C. O. Tacket, R. Edelman, and F. A. Ennis. 1993. Dengue virus-specific, human CD4<sup>+</sup> cytotoxic T lymphocytes generated in short term culture. *Viral Immunol.* 6:143-151.

## Processing of Flavivirus Structural Glycoproteins: Stable Membrane Insertion of Premembrane Requires the Envelope Signal Peptide

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The flavivirus structural proteins capsid (C), premembrane (prM), and envelope (E) are cleaved in that order from the N-terminus of the polyprotein by the ER intraluminal enzyme signal peptidase. The prM-E and E-NS1 junctions contain hydrophobic domains with both transmembrane and signal function. These domains reside at the C-termini of prM and E, respectively, after cleavage. We studied the functions of the 37-amino-acid C-terminus of the dengue virus type 4 (DEN4) prM (amino acids 243-279 of the DEN4 polyprotein) in the processing of prM and E. Hydrophobicity in this domain is interrupted by a conserved Arg residue (Arg-264) within a short amphipathic segment. Hydrophobic amino acids upstream from Arg-264 (aa 243-263) were presumed to constitute the membrane anchor for prM (the "tm" segment). Previous results had suggested that sequences downstream from Arg-264 (aa 265-279) constitute the E signal peptide. RNA transcripts prepared from wild-type (wt) and deletion-mutant DEN4 cDNAs encoding the prM signal peptide, prM, E, and the N-terminus of the nonstructural glycoprotein, NS1, were translated in rabbit reticulocyte lysate in the presence of microsomes. Processing of wt prM and E *in vitro* appeared to mimic processing occurring during flavivirus infection. Analysis of mutants confirmed the localization of the E signal peptide within residues 265 to 279. However, deletions within either the E signal peptide or the tm segment resulted in a defect in both membrane insertion of prM and cleavage of the prM-E junction. Membrane anchoring of prM appeared to be a two-step process requiring function of both the tm segment and the E signal peptide, and fully efficient prM-E cleavage was also dependent upon the integrity of both hydrophobic domains. We propose a model for the processing of the flavivirus structural glycoproteins based on these results. © 1994 Academic Press, Inc.

### INTRODUCTION

The flavivirus polyprotein is cleaved to derive the viral structural proteins capsid (C), premembrane (prM), and envelope (E), and the nonstructural (NS) proteins, in the order: C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 (reviewed in Chambers *et al.*, 1990a). Cleavages of the polyprotein at the C-prM, prM-E, E-NS1, and probably at the NS4A-NS4B sites are mediated by the host cell enzyme signal peptidase (signalase) (Chambers *et al.*, 1990b; Markoff, 1989; Nowak *et al.*, 1989; Ruiz-Linares *et al.*, 1989). Thus, the C-prM, prM-E, and E-NS1 junctions include domains with signal sequence function. A signal sequence (or signal peptide, or leader peptide) is a typically N-terminal hydrophobic sequence that targets a protein for translocation into the ER (Blobel, 1980). After translocation is initiated, a signal sequence may be cleaved by signalase in the lumen of the ER, if the signal contains the requisite cleavage site amino acids at its C-terminus (von Heijne, 1986).

Recognition of the signal sequence at the N-terminus of prM (the C-prM junction) results in initiation of the cotranslational translocation of prM as well as in cleavage at the prM N-terminus (Markoff, 1989). For pro-

cessing to proceed further, function of the structurally conserved hydrophobic sequence at the C-terminus of prM (see Fig. 1; the prM-E junction) is required: (1) Translocation of the prM moiety in the polyprotein must be arrested, and prM must be stably inserted in the membrane. These are functions of a transmembrane domain that depend on its hydrophobicity (Kyte and Doolittle, 1982) and length (Davis and Model, 1985; Haeuptle *et al.*, 1989). (2) In addition, translocation of E sequences C-terminal to prM in the polyprotein must be reinitiated, and the prM-E junction must be made available for cleavage by signalase, reiterating the functions of a signal sequence. Processing of E similarly must depend upon function of the structurally conserved hydrophobic domain at its C-terminus (the E-NS1 junction) with the result that prM and E are N-glycosylated and inserted in the membrane in a Type I ( $N_{\text{exo}}C_{\text{cyto}}$ ) orientation (von Heijne, 1988; Nowak and Wengler, 1987; Nowak *et al.*, 1989; reviewed in Chambers *et al.*, 1990a).

The C-terminal hydrophobic sequence in the dengue virus type 4 (DEN4) prM (Zhao *et al.*, 1986; see Fig. 1) is typical of that found in prM for all other flavivirus species (reviewed in Chambers *et al.*, 1990a). It is 37 amino acids in length (amino acids 243 through 279 of the DEN4 polyprotein), and its hydrophobicity is interrupted by an amphipathic segment (aa 259-264) containing a single

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conserved Arg residue (Arg-264). Hydrophobic residues upstream from Arg-264 (aa 243–263; referred to as the “tm” segment) are generally thought to constitute the transmembrane domain for prM. Residues downstream from Arg-264 (aa 265–279) constitute the putative E signal sequence (Despres *et al.*, 1990; Ruiz-Linares *et al.*, 1989). A requirement for the amphipathic segment or for conserved Arg-264 in prM has not been determined. However, the analogous amphipathic segment within the hydrophobic C-terminus of the West Nile virus E has been proposed to form a loop on the cytoplasmic side of the ER membrane, opposite the major ectodomain of E (Nowak and Wengler, 1987). Formation of the loop would require that both hydrophobic sequences that flank the amphipathic segment are intramembraneous after processing.

The mechanisms by which the component segments of the hydrophobic domain at the C-terminus of prM act in concert to effect processing of prM and E have not been previously examined. This was the goal of the present study. Mutations were introduced into the targeted region of prM nucleotide sequences in a DEN4 cDNA construct encoding the N-terminal signal sequence for prM (14 amino acids), prM (166 amino acids), E (495 amino acids), and the N-terminal 250 amino acids of NS1. Wild-type (wt) and mutant DEN4 cDNAs were transcribed, and RNA was translated *in vitro* in the presence of microsomes. Analysis of the processing of the wt and mutant polypeptides demonstrated the interdependence of the hydrophobic domains flanking the conserved Arg-264 for membrane anchoring of prM and for E signal function. Both the tm segment and the E signal sequence were required for fully efficient arrest of the translocation of prM and its stable membrane integration, as well as for cleavage at the prM–E site. In addition, conserved Arg-264 in the context of the short amphipathic segment was not required for proper processing of prM and E. A model for the processing of prM and E in which membrane anchoring of prM is a two-step event, including both the transient arrest of the translocation of prM mediated by the tm segment and subsequent recognition of the E signal sequence, is proposed.

## MATERIALS AND METHODS

### Construction of recombinant DNA

The genome of DEN4 virus, strain 814669, had previously been cloned and completely sequenced (Zhao *et al.*, 1986; Mackow *et al.*, 1987). A 4-kb cDNA bounded by *Bgl*II restriction endonuclease sites at nt 88 and 4128, including the 3'-terminal 13 nucleotides of the 5'-noncoding region and the 5'-terminus of the long ORF encoding C, prM, E, NS1, NS2A, and the first residue of NS2B, was the gift of Dr. Ching-Juh Lai. This cDNA was cloned into the phagemid vector pTZ18U (U.S. Biochemical) at the *Bam*HI site in the polylinker (pTZ-D4Kb). pTZ-Sal DNA

was generated from pTZ-D4Kb DNA by methods previously described (Markoff, 1989). pTZ-Sal DNA contains nt 88 to 999 of DEN4 cDNA and was used in the present study as a template for site-directed mutagenesis. The *Sal*I site in DEN4 DNA occurs at nt 999 to 1004. *Sal*I cuts within the codon for aa 21 of the E sequence (aa 300 of the polyprotein).

To construct recombinant DNA for transcription/translation, pTZ-D4Kb DNA was digested with the restriction endonuclease *Apa*I which cut the recombinant DNA after nt 3168 of DEN4 cDNA, within the NS1 gene sequence in the long ORF. A *Pst*I site was added to *Apa*I-linearized recombinant DNA by linker ligation. The product was digested with *Pst*I to remove DEN4 cDNA extending from nt 3168 to the preexisting *Pst*I site in the downstream pTZ polylinker and religated (pTZ-Apa DNA). Next, the 5'-end of the DEN4 cDNA insert in pTZ-Apa DNA was modified by use of the polymerase chain reaction (PCR). The 5' primer for PCR (D171; AAAGGTACCACCATGGGGTCAACGATAACATTGCTGTGC) encoded a *Kpn*I recognition sequence, a favorable sequence for translation initiation (underlined; Kozak, 1986), the added codon GGG, and the first 21 nt encoding the N-terminus of the signal peptide for prM, which initiates at nt 399 of DEN4 cDNA (Zhao *et al.*, 1986). The 3' primer for PCR (D172; TAGGTGACCCATGCTCCACCTGA) was complementary to cDNA sequences proximal to the *Sal*I site (nt 999) in the DEN4 sequence.

The PCR product and pTZ-Apa DNAs were each digested with *Kpn*I and *Sal*I. The larger fragment of the resultant digested pTZ-Apa recombinant DNA, containing DEN4 nt 1000 to 3168 and pTZ vector DNA, was selected and ligated to the *Kpn*I/*Sal*I-digested PCR product DNA. Finally, the modified DEN4 Apa DNA insert, containing the appended translational start site and DEN4 nt 399 to 3168 (see Fig. 1), was excised from pTZ vector DNA by digestion of the recombinant with *Kpn*I and *Pst*I, selected, and recloned into the phagemid vector pGEM 3zf(+) (Promega) using the homologous cloning sites in the pGEM polylinker (pGWt DNA). All oligonucleotide primers used in this and subsequent procedures were prepared using a PCR-Mate Model 391 oligosynthesizer (Applied Biosystems). All PCR reactions were conducted in a Perkin-Elmer-Cetus DNA thermal cycler using commercially available reagents.

### Mutagenesis of DEN4 cDNA

(See Fig. 1 for a diagrammatic representation of mutant constructs.) Mutations  $\Delta$ tm and  $\Delta$ Ess(2R) were initially created in pTZ-Sal DNA by site-directed mutagenesis (Zoller and Smith, 1983). The *Kpn*I-to-*Sal*I segments of  $\Delta$ tm and  $\Delta$ Ess[2R] mutant pTZ-Sal DNAs (containing the respective mutations) were then selectively amplified by PCR, using primers D171 and D172. PCR product cDNAs were in each case used to replace the wt *Kpn*I-to-*Sal*I

fragment of DEN4 cDNA in the pGWT construct. Mutations  $\Delta$ Ess(1R) and  $\Delta$ Essdr were generated from  $\Delta$ Ess(2R) DNA using PCR by taking advantage of a unique *Bgl*II site introduced into  $\Delta$ Ess(2R) DNA during site-directed mutagenesis. The *Bgl*II recognition sequence (AGATCT) encoded the amino acids (Arg-Ser) inserted into the  $\Delta$ Ess(2R) sequence downstream from conserved Arg-264 (Fig. 1). Two different negative-sense PCR primers containing 5'-*Bam*H1 sites (GGATCC, encoding Gly-Ser) in combination with primer D171 were used to synthesize *Kpn*I-to-*Bam*H1 fragments encoding respectively the  $\Delta$ Ess(1R) and  $\Delta$ Essdr mutations.  $\Delta$ Ess(2R) DNA was digested with *Kpn*I and *Bgl*II, and the excised *Kpn*I-*Bgl*II fragment was then replaced by the *Kpn*I-*Bam*H1 fragments containing the new mutations (Fig. 1). Thus, the Arg-Ser substitution encoded by  $\Delta$ Ess(2R) DNA is replaced by Gly-Ser in the  $\Delta$ Ess(1R) and  $\Delta$ Essdr mutations.

Similarly, the  $\Delta$ tmr mutation was introduced into  $\Delta$ tm DNA by PCR. Opposite-sense, overlapping primers were synthesized that each contained a *Bam*H1 recognition sequence (GGATCC) such that the codon for Arg-264 was replaced by a codon for Gly (underlined) in the *Bam*H1 recognition sequence. These primers were used separately in PCR reactions to generate fragments extending upstream to the *Kpn*I site in  $\Delta$ tm DNA and downstream to a unique *Nsi*I site at nt 1720 in the DEN4 sequence. *Kpn*I-*Bam*H1 and *Bam*H1-*Nsi*I PCR fragments were ligated to each other. The resulting *Kpn*I-*Nsi*I fragment was digested with *Sal*I and used to replace *Kpn*I-*Sal*I sequences in  $\Delta$ tm DNA in order to generate  $\Delta$ tmr mutant DNA. Mutations  $\Delta$ R and  $\Delta$ QQR were generated from wt DNA by an analogous strategy involving the generation of a *Kpn*I-*Nsi*I fragment containing the needed mutations. In this case, wt nt 883-888 (GAATCC) were altered to produce a *Bam*H1 recognition sequence (GGATCC), a silent mutation. To produce the  $\Delta$ R mutation, the nt sequence of the (+)-sense primer was additionally altered from that of wt to delete the codon for Arg-264 (CGA; nt 891-893). To produce the  $\Delta$ QQR mutation, the nt sequence of the (+)-sense primer was altered from wt such that the codon for Arg-264 was deleted and the codon for Gln-263 was replaced by a codon for Leu. That of the (-)-sense primer was altered from wt to replace the codon for Gln-259 with a codon for Leu. Nucleotide sequencing of wt and mutant DNAs within the region bounded by *Kpn*I and *Sal*I sites was performed using Sequenase (U.S. Biochemical). In addition to confirming the sequences of mutant DNAs, sequencing also revealed that DEN4 cDNA contained a codon for Gly (GGA; nt 834-836) for aa 245 (Fig. 1), rather than one for Arg (AGA), in contradiction to the previously published sequence of this DNA (Zhao *et al.*, 1986).

#### Transcription of wt and mutant recombinant DNA

Wt and mutant DEN4 DNAs were cloned downstream from the T7 RNA polymerase promoter in pGEM 3zf(+).

For transcription, DNAs were linearized either at a unique *Hind*III site in the vector polylinker downstream from DEN4 DNA inserts (*Hind*III transcripts) or at the unique *Nsi*I site in DEN4 DNA at nt 1720 (*Nsi*I transcripts). Conditions for transcription of DEN4 cDNA were previously reported (Markoff, 1989). An amount of the completed reaction estimated to contain 1 to 3  $\mu$ g of product RNA was used directly for translation.

#### Translation of RNA transcripts

Reactions contained 1 to 2  $\mu$ l of RNA transcription reaction (1-3  $\mu$ g RNA), 0.5  $\mu$ l RNasin (Promega), 1.8  $\mu$ l canine pancreatic microsomes (Promega), 0.5  $\mu$ l of 1.0 mM amino acids minus methionine (Promega), and 2.5  $\mu$ l [ $^{35}$ S]methionine (>800 Ci/mmol; 15 mCi/ml; Amersham) plus 17.5  $\mu$ l micrococcal-nuclease-treated rabbit reticulocyte lysate (Promega). Translation was carried out for 60 min at 30°. After translation, the lysate was centrifuged at 4° to pellet membranes (5 min, 10K rpm), which were then washed in 0.1 ml ice-cold phosphate-buffered saline (PBS) and repelleted prior to further treatment.

#### Deglycosylation of *in vitro*-synthesized proteins

Pelleted membrane-associated proteins were resuspended in 20  $\mu$ l endo F buffer (50 mM sodium phosphate, pH 7, 50 mM EDTA, 0.5% NP-40, 0.1% SDS, 1.0%  $\beta$ -mercaptoethanol) and incubated at 37° for 2 to 16 hr with 0.2 to 0.4 units of the enzyme endoglycosidase F (endo F; Boehringer-Mannheim).

#### Radioimmune precipitation

Peptides representing the N-terminal 15 amino acids of the DEN4 prM (FSLSTRDGEPLMIVA-C) or E (MRCVGVGNRDFVEGV) were conjugated to keyhole-limpet hemocyanin and used to immunize rabbits. The resultant antisera each had an ELISA titer against the respective immunizing peptide of >1:800. Pelleted membrane-associated proteins were resuspended in 50 to 100  $\mu$ l RIPA buffer (150 mM NaCl, 100 mM Tris-HCl, pH 7.4, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate). Rabbit antisera was added to a final dilution of 1:5 to 1:20, and the mixture was incubated at 4° for 2 to 16 hr. Antigen-antibody complexes were collected on Staphylococcal protein A using Pansorbin (Calbiochem).

#### Protease digestion of membrane-associated proteins

Pelleted membranes were resuspended in 50  $\mu$ l of ice-cold thermolysin buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM CaCl<sub>2</sub>). Thermolysin (4  $\mu$ l of a 1 mg/ml solution; Boehringer-Mannheim) was added, and reactions were incubated for 1 to 4 hr at 4°. Proteolysis was arrested by the addition of EDTA to a 50 mM final concentration, and membranes were pelleted and

washed in calcium-free buffer prior to electrophoretic analysis.

### Elution of proteins from membranes using alkali

Membranes resuspended in PBS were aliquoted, pelleted, and resuspended in either 20  $\mu$ l of 100 mM sodium carbonate, pH 11.5, or 20  $\mu$ l PBS, and incubated for 10 min at 4°, according to a method previously described (Fujiki *et al.*, 1982). Membranes were then pelleted from suspension as described, and both supernatant and pelleted fractions were saved for electrophoretic analysis. The association of proteins with membrane or supernatant fractions after alkali exposure of membranes was calculated by computer-based analysis of a phosphorimage of the dried gel (Molecular Dynamics), except where noted.

### Protein electrophoresis

Pelleted samples or Pansorbin-bound antigen/antibody complexes were suspended in Tricine sample buffer. Supernatant fractions were mixed with an equal volume of 2X Tricine sample buffer. All samples were then heated to 80° or boiled for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), in 16% gels employing a Tricine-based buffer system (Schagger and von Jagow, 1987). Electrophoresis was carried out for 15 hr at 115 V in gels of 1.5 mm thickness. After fixing in 40% methanol/10% acetic acid, gels were fluorographed, dried, and used to expose Kodak XAR-2 film.

## RESULTS

### Processing of the wt polypeptide

pGWt DNA (Wild type; Fig. 1) contained a start codon in a favorable context for translation initiation (Kozak, 1986) followed by a codon (GGG) for Gly and DEN4 nt 399 through 3168, downstream from the T7 RNA polymerase promoter. DEN4 cDNA encoded the signal peptide for prM (aa 100–113 of the polyprotein), prM (aa 114–279), E (aa 280–773), and the N-terminal 250 residues of NS1 (aa 774–1023). Each of the DEN4 glycoproteins contained two potential N-linked glycosylation sites of the Asn-X-Ser, Thr, or Cys type (Bause and Legler, 1981).

SDS-PAGE of the products of translation of wt *HindIII* RNA transcripts is shown in Fig. 2A, lane 1. The [<sup>35</sup>S]-methionine-labeled proteins were tentatively identified as mono- and diglycosylated forms of prM, E, and truncated NS1 (NS1\*), as indicated, on the basis of their relative sizes. An additional high molecular weight product migrating near the origin of the gel appeared to represent the uncleaved prM-E-NS1\* precursor. Radiolabel at the origin of some lanes in Fig. 2A (and other gels) represents proteins trapped at the interface of the separating gel and the stacking gel. PrM and E were also

formally identified by immune precipitation using rabbit anti-peptide antibodies specific for the N-terminal 15 amino acids of the DEN4 prM and E, respectively (Fig. 2B, lanes 1–3). Cleavages at the prM-E and E-NS1 sites were dependent upon the presence of microsomes during translation (data not shown). This was consistent with previous results demonstrating that these cleavages are signalase-mediated (Markoff, 1989; Despres *et al.*, 1990).

PrM, E, and NS1\* were associated with the pelletable fraction of the translation reaction, indicating they were associated with membranes. To establish their membrane orientation, they were subjected to digestion with the protease, thermolysin. All species of prM, E, and NS1\* were completely resistant to proteolysis (Fig. 2A, lane 2). In contrast, these proteins could be rendered thermolysin-sensitive, if membranes were disrupted with detergent prior to proteolysis (Fig. 2C, lane 1). Therefore, the major ectodomains of prM, E, and NS1\* were intraluminal after translocation.

To confirm identification of cleavage products by size and to demonstrate glycosylation, intraluminal (protease-resistant) proteins were digested with endo F after membranes were solubilized (Fig. 2A, lane 3). This procedure yielded three major products with the expected sizes of unglycosylated prM (expected  $M_r$  ~18 kDa; after cleavage of its 14-amino-acid signal peptide), NS1\* (expected  $M_r$  ~28 kDa), and E or monoglycosylated E (expected  $M_r$  ~54 kDa or ~57 kDa, respectively). Comparison of the sizes of translation products before and after deglycosylation (Fig. 2A, lanes 2 and 3) provided confirming evidence that each of the two N-linked glycosylation sites in prM, E, and NS1\* could be recognized *in vitro*; glycosylated species bearing either one or two mannose-rich carbohydrate moieties of expected  $M_r$  ~3 kDa each could be detected for each of the viral proteins.

### Processing of the $\Delta$ tm mutant polypeptide

We next sought to assess the function of the putative transmembrane domain (the tm segment) within the hydrophobic C-terminus of prM (aa 243–263; Fig. 1). A transmembrane domain must be able to effect both a halt in translocation (stop-transfer) and stable membrane integration (Davis *et al.*, 1985; reviewed in Blobel, 1980; von Heijne, 1988). The mutant  $\Delta$ tm polypeptide lacked an internal 16 hydrophobic amino acids (aa 247–262) of this segment (Fig. 1). If the tm segment were a necessary part of the transmembrane domain in prM, we anticipated that  $\Delta$ tm mutant prM would be defective in one or both of these functions. Results were consistent with this assumption.

Translation of *HindIII* RNA transcripts bearing the  $\Delta$ tm mutation (Fig. 2A, lane 4) resulted in the generation of cleaved and glycosylated NS1\* and in small amounts of properly processed E and prM. In addition, a group of

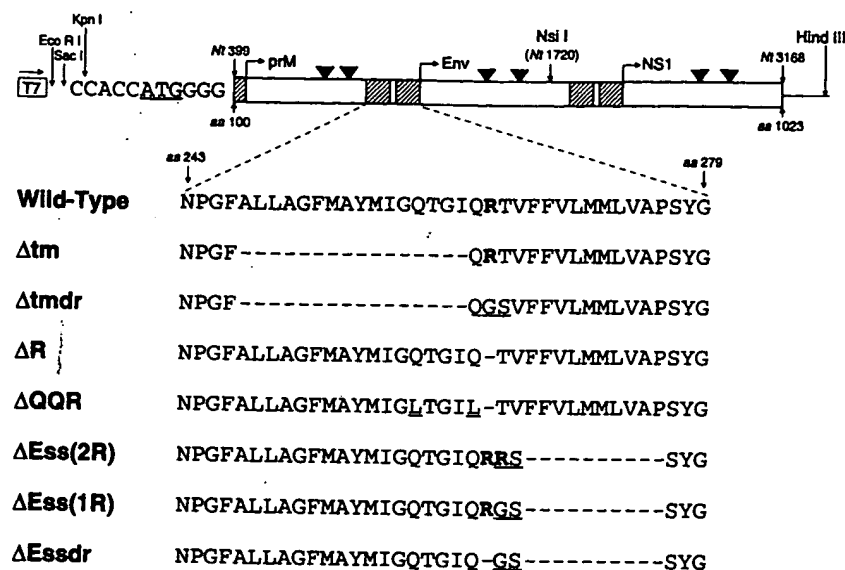


Fig. 1. Mutations introduced into the hydrophobic C-terminus of the dengue virus prM. The T7 RNA polymerase promoter (T7), the 5'-polylinker region, and cloned DEN4 cDNA in recombinant pGWT DNA are depicted across the top. Rectangles denote both the T7 promoter sequence and DEN4 cDNA nucleotides (Nt) 399 to 3168, encoding prM, the envelope glycoprotein (Env), and the N-terminus of NS1, amino acids (aa) 100 to 1023. The horizontal arrow above the T7 promoter segment indicates the direction of transcription. The last two nucleotides of the KpnI site in pGEM upstream from DEN4 cDNA and nucleotides added to the 5' end of DEN4 cDNA by PCR, prior to cloning at the KpnI site in the pGEM polylinker, are shown; the appended start codon is underlined. Recombinant DNA was linearized for RNA transcription at either the HindIII site in the pGEM polylinker downstream from DEN4 cDNA or the NsiI site within DEN4 cDNA sequences encoding Env. Signalase cleavage sites for the N-termini of prM, Env, and NS1 are indicated by right-angled arrows. The approximate locations of Asn-linked glycosylation sites are indicated by closed inverted triangles. Hydrophobic domains within the DEN4 polyprotein are indicated by shaded areas. The wild-type amino acid sequence of the hydrophobic C-terminus of prM (aa 243 to 279) is shown, using the single letter amino acid code. The amino acid sequences in mutant polyproteins Δtm, Δtm<sup>dr</sup>, ΔR, ΔQQR, ΔEss(2R), ΔEss(1R), and ΔEss<sup>dr</sup> are aligned with that of wt. Deletions of wt amino acids in mutant sequences are indicated by dashes. Missense mutations are indicated by underlining. Positively charged Arg residues are indicated in bold print.

high molecular weight proteins migrating in the 72- to 78-kDa size range was generated. These proteins were the expected sizes of glycosylated forms of uncleaved prM+E molecules. They were subsequently identified as such by immune precipitation using the anti-peptide antibodies specific for the N-termini of prM and E, respectively (Fig. 2B, lanes 4-6).

To assess the membrane orientation of Δtm mutant proteins, the membrane fraction of the translation reaction was subjected to proteolysis, as previously described. The majority of Δtm prM+E chimeric molecules were completely resistant to proteolysis, indicating that they were wholly intraluminal (Fig. 2A, lane 5). Cleaved prM bearing the Δtm mutation, E, and NS1\* also resisted proteolysis and therefore appeared to present their ectodomains on the luminal side of the ER membrane. Protease treatment also consistently resulted in a slight increase in intensity of bands of the approximate size of glycosylated prM and a concomitant slight decrease in the intensity of bands representing chimeric prM+E molecules (Fig. 2A, lanes 4 and 5; best seen in Fig. 3A, lanes 1 and 2). This suggested that a small proportion of Δtm chimeric prM+E molecules had attained a transmembrane prM<sub>lumen</sub>E<sub>cyto</sub> orientation, rendering the E moiety sensitive to protease. After proteolysis, these membrane-spanning chimeric molecules were reduced in size to

approximately that of prM. Consistent with this analysis, deglycosylation of protease-resistant Δtm polypeptides yielded proteins of the expected sizes of unglycosylated prM, E, NS1\*, and prM+E (Fig. 2A, lane 6).

In summary (see Table 1), the Δtm mutation had a major negative effect on stop-transfer of the prM moiety, as expected. This was evident in the accumulation of the majority of prM+E chimeric molecules in the ER lumen. Stop-transfer of prM in the small proportion of chimeric molecules that attained a transmembrane orientation most likely was effected by hydrophobic residues, including the E signal peptide, remaining at the C-terminus of Δtm mutant prM (see Fig. 1). (Scanning of the amino acid sequence of the DEN4 prM and E [Zhao *et al.*, 1986] revealed no other candidate membrane anchor domain in the vicinity of the C-terminus of prM.) In addition, the Δtm mutation resulted in a defect in cleavage of the prM-E site, relative to wt. This was most likely secondary to the failure of the mutant tm segment to arrest translocation, as will be discussed. Assessment of the effect of the Δtm mutation on the stability of membrane integration of prM is the subject of additional experiments (see below).

#### Processing of the ΔEss(2R) polypeptide

The role of the putative E signal peptide (aa 265-279) in processing of prM and E was next determined. Mutant

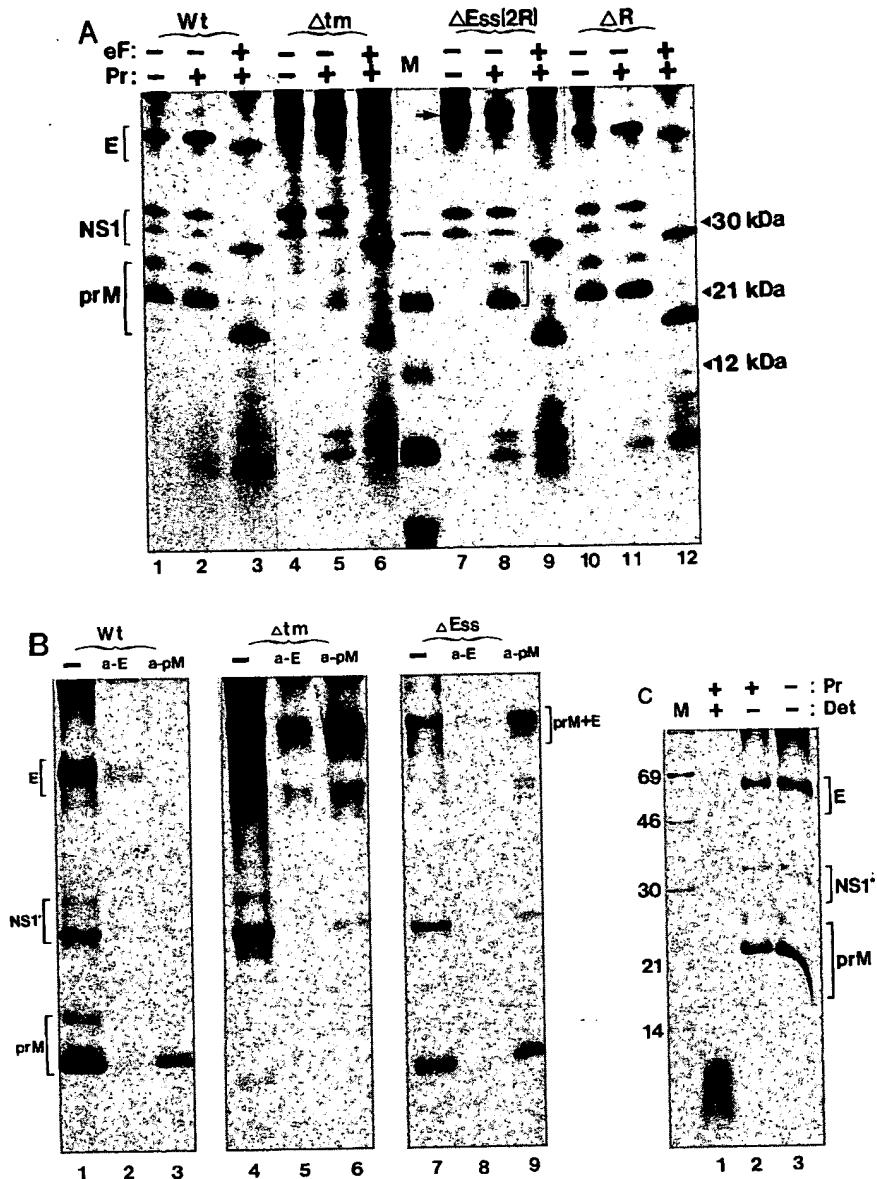


FIG. 2. (A) Translation of wt and mutant RNAs prepared from  $\phi$ g recombinant DNAs linearized at the *Hind*III site. Wild type (lanes 1–3) and mutant RNAs  $\Delta$ tm (lanes 4–6),  $\Delta$ Ess(2R) (lanes 7–9), and  $\Delta$ R (lanes 10–12) were translated in the presence of added canine pancreatic microsomes and [ $^{35}$ S]methionine. Membranes were pelleted from suspension and washed once in cold thermolysin buffer. Approximately one-third of the washed membranes were untreated prior to electrophoresis (lanes 1, 4, 7, and 10). The remaining two-thirds of the membrane-associated proteins were digested with thermolysin. An excess of EDTA was then added, and the thermolysin-digested fractions were divided into two aliquots. One aliquot was saved for electrophoresis (lanes 2, 5, 8, and 11), and the other was pelleted and resuspended in endo F buffer prior to digestion with endoglycosidase F (lanes 3, 6, 9, and 12). Samples were electrophoresed in an SDS–16% polyacrylamide–Tricine-buffered gel. Glycosylated and deglycosylated forms of prM, E, and NS1 cleaved from the encoded polyprotein are bracketed to the left of the gel. An arrow indicates the position in the gel of uncleaved prM+E chimeric molecules resulting from defective processing of the mutant  $\Delta$ Ess(2R) polypeptide (lanes 7 and 8). The analogous product is also seen after processing of the  $\Delta$ tm mutant polypeptide (lanes 4 and 5). The prM-sized product of proteolysis of  $\Delta$ Ess(2R) prM+E molecules is indicated by a bracket to the right of lane 8. eF, endoglycosidase F; Pr, protease (thermolysin); (–), not digested; (+), digested. (B) Immune precipitation of the products of cell-free translation. RNA transcripts of wt,  $\Delta$ Ess(2R), and  $\Delta$ tm cDNAs linearized at the *Hind*III site were translated, and microsomal membranes were isolated. Membrane-associated proteins were subjected to proteolysis using thermolysin. Subsequently, wt (lanes 1–3),  $\Delta$ tm (lanes 4–6), and  $\Delta$ Ess(2R) (lanes 7–9) proteins were solubilized in RIPA buffer and immune-precipitated using sera derived from rabbits immunized with KLH-conjugated 15-amino-acid peptides specific for the amino-termini of the DEN4 E (a-E; lanes 2, 5, 8) and prM (a-pM; lanes 3, 6, 9), respectively. Total recovered products of translation of wt,  $\Delta$ tm, and  $\Delta$ Ess(2R) transcripts are shown in lanes 1, 4, and 7, respectively (labeled “–”). Proteins were separated on an SDS–16% polyacrylamide–Tricine-buffered gel. The locations in the gel of prM+E chimeric molecules, authentic prM and prM-sized products of the proteolysis of membrane-spanning  $\Delta$ Ess(2R) chimeric molecules (“prM”), and E, are indicated. A small fraction of the [ $^{35}$ S]methionine-labeled proteins identified as glycosylated forms of NS1 in Fig. 2A and elsewhere were a consistent contaminant of a-pM antibody immune precipitates. (C) Detergent renders wt proteins in membranes susceptible to proteolysis. Cell-free translation of wt *Hind*III RNA transcripts was conducted. Membranes containing radiolabeled proteins were washed and resuspended in thermolysin buffer, and the suspension was divided into three aliquots. One aliquot was digested with thermolysin in the presence of 2%  $\beta$ -octylglucoside (+Pr, +Det; lane 1). A second aliquot was digested with thermolysin in the absence of detergent (+Pr, –Det; lane 2), and the third aliquot was untreated (–Pr, –Det; lane 3).

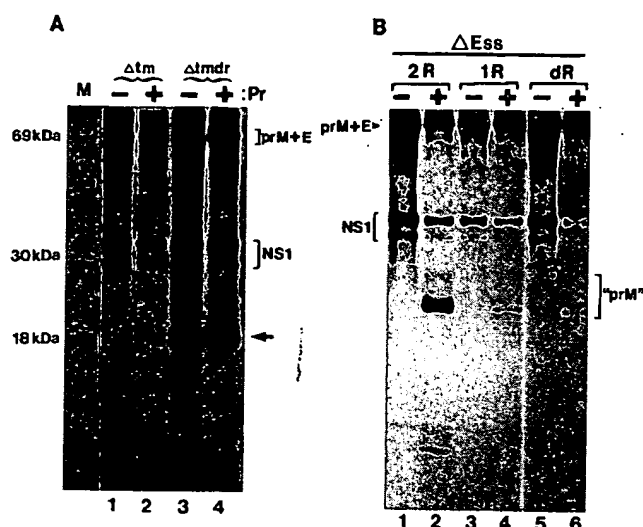


Fig. 3. Sensitivity to protease of membrane-associated mutant polypeptides. RNA transcripts were prepared from mutant recombinant DNAs that had been linearized by digestion at the *Hind*III site (see the legend to Fig. 1). RNA was translated in rabbit reticulocyte lysate in the presence of canine pancreatic microsomes, and membranes were pelleted by centrifugation and resuspended in thermolysin buffer. The suspension was divided into two aliquots, and one aliquot was subjected to digestion by thermolysin. Membranes were then pelleted from both aliquots, and proteins were solubilized by resuspension in gel sample buffer prior to electrophoresis. Pr, protease; (-), not digested; (+), digested. (A) Results of protease digestion of membrane-associated  $\Delta$ tm (lanes 1 and 2) and  $\Delta$ tmdr (lanes 3 and 4) *Hind*III polypeptides are compared. An arrow indicates the position of the prM-sized moiety resulting from proteolysis. (B) Results of protease digestion of membrane-associated  $\Delta$ Ess(2R) (lanes 1 and 2), (1R) (lanes 3 and 4), and dR (lanes 5 and 6) *Hind*III polypeptides are compared. The location of the prM-sized moiety derived from the proteolysis of  $\Delta$ Ess mutants ("prM") is indicated.

$\Delta$ Ess(2R) had a substitution of Arg-Ser for 12 (aa 265-276) of the 15 amino acids that constitute this domain (Fig. 1). Thus, the hydrophobic 10-amino-acid core of the putative signal was deleted in  $\Delta$ Ess mutants; however, the predicted signalase cleavage site at the prM-E junction was left intact. This site consists of the small hydrophobic residues in the -3 to -1 positions with respect to the predicted N-terminus of E (Ser<sub>277</sub>-X-Gly<sub>279</sub>; von Heijne, 1986). Therefore, reinitiation of the translocation of E (E signal function) would be indicated by cleavage of the prM-E site.

Translation of mutant  $\Delta$ Ess(2R) *Hind*III RNA transcripts (Fig. 2A, lane 7) resulted primarily in the production of the glycosylated forms of NS1\* and a family of proteins in the 72- to 78-kDa size range. These were identified as glycosylated forms of prM+E chimeric molecules by immune precipitation using both prM-specific and E-specific rabbit anti-peptide antibodies (Fig. 2B, lanes 7-9). Faint bands of the approximate sizes of mono- and diglycosylated E were also noted (Fig. 2A, lane 7, compare to lane 1). However, since proteins of the expected sizes of cleaved and glycosylated prM were not detected at

the level of sensitivity of these experiments (Fig. 2A, lane 7), we assumed that the small amount of E-sized proteins generated were nonspecific products of internal initiation or premature termination of translation. Thus, we concluded that the  $\Delta$ Ess[2R] mutation abrogated cleavage of the prM-E site (see Table 1). This indicated that hydrophobic amino acids deleted from the  $\Delta$ Ess(2R) mutant were required for E signal function.

Translocation of chimeric  $\Delta$ Ess(2R) prM+E molecules was directly assessed by thermolysin digestion of translation products in intact membranes (Fig. 2A, lane 8), as was done for wt and  $\Delta$ tm products. This analysis revealed that the E moiety in a major fraction of chimeric molecules was thermolysin-sensitive; proteolysis of this fraction resulted in the generation of proteins of the sizes of mono- and diglycosylated prM ("prM", bracketed in Fig. 2A, lane 8). This tentative identification was subsequently confirmed, since deglycosylation of these proteins yielded a product which approximately comigrated with deglycosylated prM (Fig. 2A, compare lane 3 to lane 9). In addition, the major glycosylated prM-sized product was immune precipitated by prM-specific anti-peptide antibodies, indicating it included the N-terminus of prM (Fig. 2B, lane 9). Therefore, the majority population of chimeric molecules had attained the transmembrane prM<sub>lumen</sub>E<sub>cyto</sub> orientation in which the hydrophobic tm segment constituted the membrane-spanning domain.

Surprisingly, a minor fraction of uncleaved prM+E molecules was completely resistant to proteolysis despite extensive exposure to thermolysin (Fig. 2A, lane 8; indicated by an arrow in lane 7), suggesting this fraction was wholly intraluminal. This indicated that the tm segment alone was not sufficient to arrest the translocation of the prM moiety; hydrophobic residues of the E signal peptide deleted in the  $\Delta$ Ess(2R) mutant polypeptides were also required for fully efficient arrest of the translocation of prM.

#### Processing of $\Delta$ R and $\Delta$ QQR mutant polypeptides

A requirement for the conserved amphipathic domain (aa 259-263) during processing of prM and E was next sought. Mutant  $\Delta$ R contained a deletion of Arg-264; mutant  $\Delta$ QQR contained a deletion of Arg-264 and substitutions of Leu for Gln-259 and Gln-263 (Fig. 1).

A comparison of the translation products of mutant  $\Delta$ R *Hind*III transcripts to those of wt in intact membranes demonstrated that the  $\Delta$ R mutation had no effect on translocation, cleavage, or glycosylation of prM, E, and NS1\* (Fig. 2A, compare lanes 1-3 to lanes 10-12). Similar analysis of proteins bearing the  $\Delta$ QQR mutation gave identical results (data not shown).

#### Processing of $\Delta$ tmdr, $\Delta$ Ess(1R), and $\Delta$ Essdr mutant polypeptides

The  $\Delta$ tmdr,  $\Delta$ Ess(1R), and  $\Delta$ Essdr mutations (Fig. 1) were constructed to determine the effect of conserved

TABLE 1  
CLEAVAGE OF THE prM-E SITE, TRANSLOCATION, AND MEMBRANE INTEGRATION OF prM

Construct	prM-E cleavage <sup>a</sup> (est. %)	Translocation <sup>b</sup>	Membrane integration of prM moiety <sup>c</sup>
Wt	>95	prM <sub>IN</sub> E (or E*) <sub>IN</sub>	Yes (defined as "stable")
Δtm	5 to 10	(1) ~80%: (prM+E) <sub>IN</sub> (2) ~10%: prM <sub>IN</sub> +E <sub>OUT</sub> (3) ~10% cleaved prM <sub>IN</sub>	No Yes, unstable
Δtm <sub>dr</sub>	5 to 10	(1) ~50%: (prM+E) <sub>IN</sub> (2) ~40%: prM <sub>IN</sub> +E <sub>OUT</sub> (3) ~10% cleaved prM <sub>IN</sub>	No Yes, unstable
ΔEss(2R)	Not detected	(1) ~75%: prM <sub>IN</sub> +E <sub>OUT</sub> (2) ~25%: (prM+E) <sub>IN</sub>	Yes No
ΔEss(1R); ΔEss <sub>dr</sub>	Not detected	(1) ~50%: prM <sub>IN</sub> +E <sub>OUT</sub> <sup>d</sup> (2) ~50%: (prM+E) <sub>IN</sub>	Yes, unstable No

<sup>a</sup> Polyproteins were derived from *in vitro* translation of wt transcripts encoding the prM signal peptide, prM, E, and the amino-terminus of NS1 (NS1\*; *Hind*III transcripts) or the prM signal peptide, prM, and the amino-terminus of E (E\*; *Nsi*I transcripts) for DEN4 virus. Processing was assessed as discussed under Materials and Methods and Results.

<sup>b</sup> The subscript "IN" indicates the protein was intraluminal in the ER, as suggested by its resistance to proteolysis in intact membranes. The subscript "OUT" indicates the protein was sensitive to proteolysis under the same conditions. The percentage of each cleavage product derived from a given construct was calculated from direct determination of radiolabel in the relevant bands in at least one experiment. "+" indicates uncleaved chimeric molecules.

<sup>c</sup> "Yes" indicates that the percentage association of the protein with membranes after alkali exposure was comparable to that of wt prM; "No" indicates that this percentage was comparable to that of E\*, which lacked the carboxy-terminal membrane anchor domain of full-length E and was taken as a negative control for membrane integration. "Unstable" indicates that the percentage association with membranes after alkali was intermediate between that of wt prM and E\*.

<sup>d</sup> 52% for ΔEss(1R) prM + E\*; 45% for ΔEss<sub>dr</sub> prM + E\*.

Arg-264 within the context of the Δtm and ΔEss mutations, respectively. Mutant Δtm<sub>dr</sub> contained the Δtm internal deletion and in addition substitutions of Gly for Arg-264 and Ser for Thr-265. The ΔEss(1R) polypeptide was similar to that of ΔEss(2R), with a substitution of Gly-Ser (instead of Arg-Ser) for residues 265 through 276, a net deletion of 10 hydrophobic amino acids in the presumptive E signal peptide. Similarly, the mutant ΔEss<sub>dr</sub> was identical to ΔEss(1R), except that Arg-264 was deleted.

A comparison of the proteins resulting from translation of Δtm and Δtm<sub>dr</sub> *Hind*III RNA transcripts is shown in Fig. 3A. Processing of the Δtm<sub>dr</sub> polypeptide was completely analogous to that of the Δtm polypeptide, with one exception. Proteolysis of Δtm<sub>dr</sub> proteins resulted in a much greater relative increase in intensity of the prM-sized protease-resistant band than did similar treatment of Δtm proteins (indicated by an arrow in Fig. 3A; compare lanes 2 and 4). This suggested that a greater fraction of Δtm<sub>dr</sub> chimeric prM+E molecules had attained the membrane-spanning prM<sub>lumen</sub>E<sub>cyto</sub> orientation than was the case for Δtm prM+E molecules. Direct analysis of duplicate experiments confirmed this finding. About 40% of Δtm<sub>dr</sub> prM+E molecules attained this orientation

versus about 10% of Δtm prM+E molecules (data not shown; see Table 1). (For "direct analysis," the relevant bands were located on the dried gel, cut out of the gel, and counted using a liquid scintillation counter.) Thus the 21-amino-acid hydrophobic C-terminus of prM in the Δtm<sub>dr</sub> mutant polypeptide was more efficient in arresting transfer of the prM moiety than the analogous hydrophobic domain in the Δtm mutant polypeptide. This difference in phenotype was probably related to the absence of the charged amino acid, Arg-264, from the Δtm<sub>dr</sub> mutant hydrophobic domain, enhancing its hydrophobicity compared to the analogous segment in the Δtm mutant (see Fig. 1).

A comparison of the proteins resulting from the translation of *Hind*III RNA transcripts encoding ΔEss(2R), (1R), and dR mutant polypeptides is shown in Fig. 3B. As for the Δtm and Δtm<sub>dr</sub> mutations, the phenotypes of the three ΔEss mutations also differed only with respect to the proportion of uncleaved prM+E molecules that attained the membrane-spanning prM<sub>lumen</sub>E<sub>cyto</sub> orientation, as revealed by proteolysis of chimeric proteins in intact membranes. The proportion of "prM" (representing the protease-resistant portion of chimeric molecules in which only prM was intraluminal) to prM+E (represent-



ing wholly intraluminal protease-resistant chimeric molecules) was evidently higher for the  $\Delta\text{Ess}(2\text{R})$  construct than for either the  $\Delta\text{Ess}(1\text{R})$  or  $\text{dr}$  constructs (Fig. 3B, lanes 2, 4, and 6). That is,  $\Delta\text{Ess}(2\text{R})$  chimeric molecules were more likely to be arrested in translocation than  $\Delta\text{Ess}(1\text{R})$  or  $\text{dr}$  chimeric molecules. Although not evident in Fig. 3B, additional experiments (not shown) revealed that  $\Delta\text{Ess}(1\text{R})$  proteins were in turn slightly more likely to be arrested in translocation than  $\Delta\text{Essdr}$  proteins. These findings were confirmed by excising the relevant bands from gels and quantifying the radiolabel present, in duplicate experiments (data not shown; see Table 1). In summary, the charged Arg residues progressively augmented arrest of the translocation of the prM moiety in the context of the  $\Delta\text{Ess}$  mutation.

### Membrane insertion of wt and mutant prM

Products of *NsII* transcripts were chosen for analysis of the relative stability of membrane integration of wt and mutant polypeptides, because truncated E ( $\text{E}^*$ ) encoded by *NsII* transcripts lacked the C-terminal hydrophobic domain in full-length E (see Fig. 1). Therefore, chimeric *NsII*-truncated proteins (prM+ $\text{E}^*$ ) contained only the single C-terminal hydrophobic domain in prM capable of mediating membrane integration, after cleavage of the prM signal peptide. Truncation of E did not alter the phenotypes of wt and mutant constructs, with the one exception noted below.

In this study, membranes containing associated radiolabeled products of translation were exposed to alkali to disrupt their closed tubular structure (Fujiki *et al.*, 1982). Membrane-associated and solubilized proteins were then separated by low-speed centrifugation. Pelletable (membrane) and supernatant fractions were subsequently analyzed by SDS-PAGE. The association of integrated proteins with the membrane fraction was expected to be relatively alkali-resistant, while that of unanchored intraluminal proteins was expected to be relatively alkali-sensitive.

**Wt prM.** Initially, aliquots of membranes bearing radiolabeled wt prM and  $\text{E}^*$  were incubated in PBS or pH 11.5-carbonate buffer to establish the specific effect of alkali exposure (Fig. 4). Protein bands representing wt prM and  $\text{E}^*$  present in membrane or supernatant fractions were excised from the gel shown, and their radioactivity was determined. The percentage of total radiolabel recovered in the membrane fraction was then calculated. Alternatively, the distribution of wt (and mutant) polypeptides between membrane and supernatant fractions was calculated by computer-based analysis of a phosphorimage. As was evident from inspection of Fig. 4, quantification of the results confirmed that PBS had little effect on the membrane association of wt prM and  $\text{E}^*$  (90% of prM and 93% of  $\text{E}^*$  remained in the membrane fraction; compare lane 1 to lane 3), whereas alkali exposure had

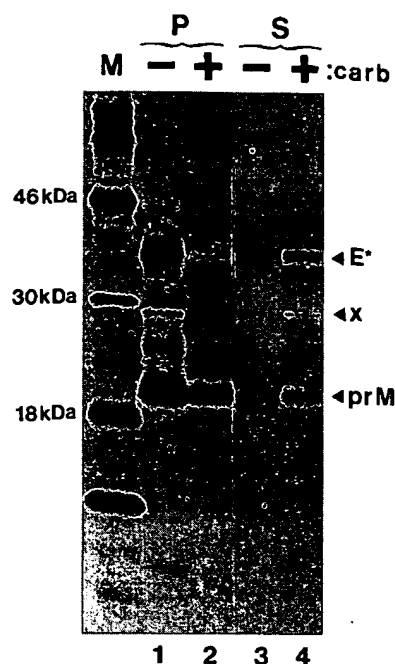


Fig. 4. Membrane integration of wt prM. Recombinant pGWT cDNA was linearized by digestion at the unique *NsII* site in the DEN4 E nt sequence, upstream from sequences encoding the transmembrane domain in E. *NsII* RNA transcripts were prepared and translated in rabbit reticulocyte lysate in the presence of canine pancreatic microsomes. Aliquots of microsomes were washed in PBS and resuspended in a pH 11.5-carbonate buffer (+) or in PBS (−). After incubation on ice, membranes were again pelleted, and [ $^{35}\text{S}$ ]methionine-labeled proteins in the resulting pellet (P) and supernatant (S) fractions were analyzed by SDS-PAGE. The positions in the gel of prM and  $\text{E}^*$  are indicated. The position of an unidentified protein (X), consistently detected after cell-free translation of *NsII* transcripts, is also indicated.

a differential effect on their disposition; a majority of prM molecules (79% of total cpm in prM) and a minority of anchorless  $\text{E}^*$  molecules (37% of total cpm in  $\text{E}^*$ ) remained associated with the membrane fraction (compare lane 2 to lane 4). These results were typical of six experiments. The percentage association of prM and  $\text{E}^*$  with membranes after alkali exposure varied among experiments, but the percentage of prM associated with membranes was approximately twice that of  $\text{E}^*$  in each case. We speculate that variation in the percentages obtained was due to the use of different microsome preparations and to their repeated freeze-thawing. Results were consistent with the concept that wt prM was membrane-anchored, whereas  $\text{E}^*$  was unanchored in the ER lumen.

**Mutant prM.** Membrane integration of the prM moiety in mutant  $\Delta\text{Ess}(2\text{R})$ ,  $\Delta\text{Essdr}$ ,  $\Delta\text{tm}$ , and  $\Delta\text{tmr}$  polypeptides was next assessed in comparison to wt prM and  $\text{E}^*$ . The wt proteins represented the positive and negative controls, respectively, for membrane insertion. To distinguish membrane-spanning from wholly intraluminal mutant chimeric molecules, proteins in intact membranes were subjected to proteolysis prior to alkali exposure (Fig. 5A). Results of an experiment in which proteins were



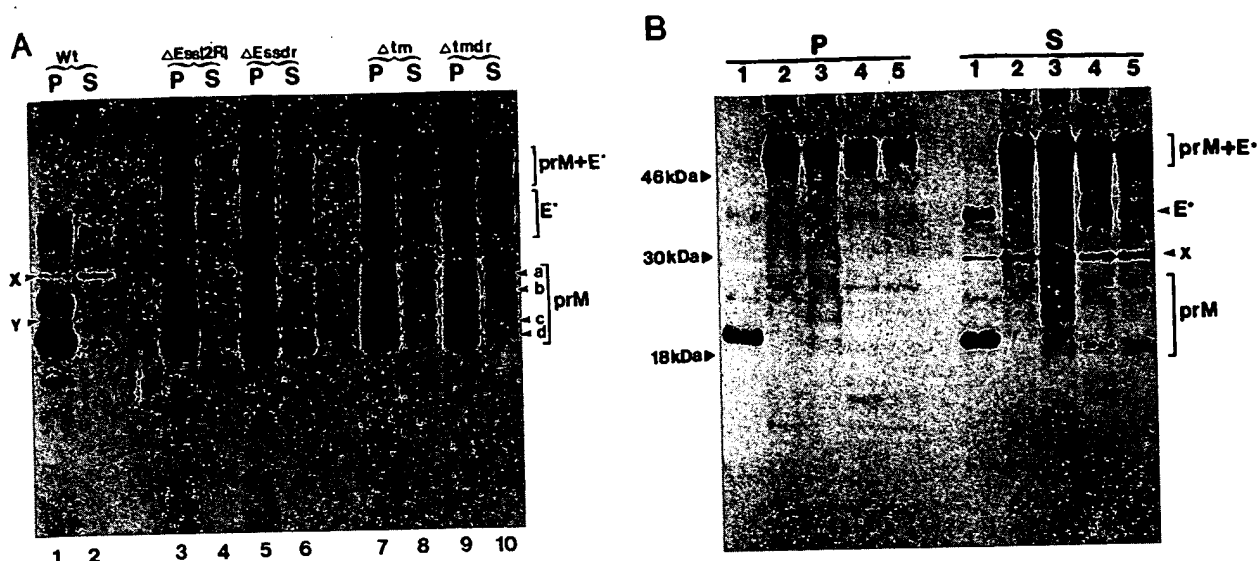


FIG. 5. (A) Elution of wt and mutant *NsII* polypeptides from membranes after proteolysis and exposure to alkali. *NsII* RNA transcripts were translated in rabbit reticulocyte lysate in the presence of canine pancreatic microsomes. Membranes were washed by centrifugation in PBS and resuspended in thermolysin buffer. After digestion by thermolysin, membranes were again pelleted and resuspended in a pH 11.5-carbonate buffer. After exposure to carbonate, membranes were again pelleted, and [<sup>35</sup>S]methionine-labeled proteins in the resulting pellet (P) and supernatant (S) fractions were separated by SDS-PAGE. The positions of unidentified proteins X and Y, cleaved prM, carboxy-terminally truncated (anchorless) E\*, and prM + E\* chimeric molecules are indicated. Di- and monoglycosylated forms of prM derived from proteolysis of membrane-spanning chimeric molecules (bands a and c, respectively) or from signalase-mediated cleavage of the prM-E site (bands b and d) are separately indicated. (B) Elution of wt and mutant *NsII* polypeptides from membranes after exposure to alkali without prior proteolysis of membrane-spanning molecules. The experiment was conducted as described for A, except digestion with thermolysin was omitted. Proteins derived from translation of Wt (lanes 1), ΔEss(2R) (lanes 2), ΔEssdr (lanes 3), Δtm (lanes 4), and Δtmldr (lanes 5) *NsII* RNA transcripts are indicated. P, pelleted (membrane) fractions. S, supernatant fractions.

not subjected to proteolysis prior to alkali exposure are also shown for comparison (Fig. 5B).

For ΔEss mutants (ΔEss(2R) and ΔEssdr), the entire population of prM-sized molecules present after proteolysis represented the protected intraluminal remnant of membrane-spanning chimeric molecules in the prM<sub>lumen</sub>-E<sub>cyto</sub> orientation (Fig. 5A, lanes 3–6), since this mutation abrogated detectable prM-E\* cleavage mediated by signal peptidase (Fig. 5B, lanes 2 and 3).

For Δtm mutants (Δtm and Δtmldr), four prM-sized proteins were detected after proteolysis (bands a, b, c, and d; Fig. 5A, lanes 7–10). Two of these proteins (bands b and d) comigrated with the small amounts of mono- and diglycosylated prM generated by signalase cleavage of the prM-E site, as seen in the absence of proteolysis (Fig. 5B, lanes 4 and 5). Therefore, proteins in bands b and d were identified as the glycosylated forms of signalase-cleaved mutant prM. The two additional prM-sized products appeared only after proteolysis (bands a and c, Fig. 5A, lanes 7–10; note their absence in Fig. 5B, lanes 4 and 5). Bands a and c were therefore taken to represent the mono- and diglycosylated intraluminal fragments of prM generated by proteolysis of membrane-spanning prM+E\* molecules. Thus, for the Δtm mutants, membrane integration of signalase-cleaved prM and membrane-spanning (prM<sub>lumen</sub>-E<sub>cyto</sub>) chimeric molecules (as well as wholly intraluminal chimeric molecules) could be separately assessed.

For the experiment shown in Fig. 5A, 96% of wt prM molecules and 58% of E\* molecules remained associated with the membrane fraction after protease treatment and alkali exposure (lanes 1 and 2). Results for mutant prM moieties in this experiment and in two additional repeat experiments were as follows (summarized in Table 1): (i) All wholly intraluminal (protease resistant) mutant chimeric prM+E\* molecules appeared to be free in the ER lumen, since their degree of association with membranes (the pellet fraction) after alkali exposure was nearly identical (±3%) to that of anchorless E\* (lanes 3–10). (ii) prM derived from proteolysis of membrane-spanning ΔEss(2R) chimeric molecules (prM<sub>lumen</sub>-E<sub>cyto</sub>) remained associated with the membrane fraction after alkali exposure in the same proportion as wt prM (±1%; lanes 3–4), our standard for "stable" membrane insertion. (iii) ΔEssdr, Δtm, and Δtmldr prM moieties, whether generated by signalase-mediated cleavage or by proteolysis of membrane-spanning chimeric molecules, were intermediate between wt prM and E\* in the percentage associated with membranes after alkali exposure (lanes 5–10). Additional experiments demonstrated a similar result for ΔEss(1R) membrane-spanning chimeric molecules (data not shown). Thus, it appeared that these mutant prM moieties were inserted in membranes, but less stably than wt prM. We concluded that the hydrophobic cores of both the tm segment and the E signal sequence were required for the stable membrane insertion of prM,

as defined by results with wt prM (Table 1). The  $\Delta\text{Ess}(2\text{R})$  mutation was an exception. In this mutant, excess positive charge (see Fig. 1) augmented the capacity of the tm segment alone to effect stable membrane insertion, as it also augmented the stop-transfer function of this segment.

In addition to prM-sized proteins, E\*-sized proteins and proteins slightly larger than E\* were generated after proteolysis of  $\Delta\text{Ess}$  mutant chimeric proteins in this experiment (bracketed "E\*" in Fig. 5A, lanes 3–6). For the  $\Delta\text{tm}$  mutants, E\*-sized proteins were observed in the absence of proteolysis (Fig. 5B, lanes 4–5), and additional proteins slightly larger than E\* were observed after proteolysis (Fig. 5A, lanes 7–10). Such proteins were never observed as products of full-length (*Hind*III) mutant constructs. We speculate that these E\*-sized proteins were generated from a population of chimeric prM+E\* molecules that initially attained a prM<sub>cyto</sub>E<sub>lumen</sub> orientation (in contrast to the prM<sub>lumen</sub>E<sub>cyto</sub> orientation routinely noted for membrane-spanning chimeric molecules). After translocation-arrest of prM<sub>cyto</sub>E<sub>lumen</sub> chimeric molecules, E\*-sized proteins could be generated by signalase cleavage of the prM-E site or by proteolysis of molecules in which prM, rather than E\*, was accessible to protease. The prM<sub>cyto</sub>E<sub>lumen</sub> orientation could result if hydrophobic residues present at the C-terminus of mutant prM were recognized as a signal peptide instead of the signal peptide at the prM N-terminus (see Fig. 1). The E\* moiety in such chimeric molecules would be initially translocated into the lumen of the ER, while mutant prM would remain cytoplasmic. In support of this hypothesis, we noted that chimeric molecules resulting from the expression of a  $\Delta\text{Ess}(1\text{R})$  mutant completely lacking the prM signal peptide uniformly attained the prM<sub>cyto</sub>E<sub>lumen</sub> orientation (data not shown).

#### Processing *in vivo*

To confirm *in vitro* observations of the phenotypes of mutant constructs, wild-type and mutant versions of a DEN4 genomic cDNA fragment encoding C, prM, E, NS1, and NS2A were expressed *in vivo* using a recombinant vaccinia virus vector. Cleavage and glycosylation of wt,  $\Delta\text{R}$ ,  $\Delta\text{Ess}(2\text{R})$ , and  $\Delta\text{tm}$  mutant polyproteins were analyzed by immune precipitation of radiolabeled infected cell extracts (data not shown). The results showed: (i) The phenotype of the  $\Delta\text{R}$  mutant was indistinguishable from wt; (ii) The  $\Delta\text{Ess}(2\text{R})$  mutation completely eliminated prM-E cleavage, resulting in expression of glycosylated uncleaved prM+E chimeric molecules; (iii) The  $\Delta\text{tm}$  mutation greatly reduced cleavage, resulting in the production of small amounts of apparently normal prM and E and larger amounts of glycosylated chimeric prM+E. The membrane orientation of the prM+E chimeras was not further investigated. All constructs expressed apparently normal NS1. These *in vivo* results were consistent with the *in vitro* results reported above.

#### DISCUSSION

We dissected *in vitro* the processing functions mediated by the hydrophobic C-terminus of the DEN4 prM after the initiation of translocation of prM. These functions include the interruption of translocation of prM and the reinitiation of translocation of the polyprotein, accompanied by prM-E cleavage and anchoring of prM in the membrane. Our analysis of the processing of wt prM, E, and NS1\* suggested the validity of the *in vitro* system for modeling the processing events that occur as a consequence of flavivirus infection. prM, E, and NS1\* were translocated, cleaved, and glycosylated following efficient recognition of the N-terminal prM signal peptide. Cleavage events were dependent upon the presence of microsomes. The data suggested that prM was membrane anchored in the predicted type I orientation (N<sub>exo</sub>C<sub>cyto</sub>), and we assume that E is also anchored in this orientation by its C-terminal hydrophobic domain, which is completely analogous in structure to that of prM. Lumenal localization of the ectodomains of the structural glycoproteins is consistent with a model for virion morphogenesis which predicts that cytoplasmic nucleocapsid structures composed of virion C and genomic RNA acquire a lipid envelope by budding into the ER (Hase *et al.*, 1987a,b; Leary and Blair, 1980). After budding in this fashion, prM and E would be displayed on the virion surface.

#### Membrane anchoring of prM

The 10-amino-acid hydrophobic core of the E signal peptide was required in addition to the central hydrophobic 16 amino acids of the tm segment for efficient arrest of the translocation of prM. Similarly, neither of these hydrophobic domains alone appeared able to effect stable membrane integration of the prM moiety in those fractions of  $\Delta\text{tm}$  and  $\Delta\text{Ess}$  chimeric molecules that were arrested in the membrane during translocation.

The failure of the tm segment to stand alone as an efficient stop-transfer/transmembrane domain is not likely to be due to insufficient length. Theory predicts that 20 hydrophobic amino acids in an  $\alpha$ -helix are sufficient to span the membrane (Eisenberg, 1984). In practice, 16 residues composed of a repeating unit of 4 hydrophobic amino acids were sufficient to anchor the coliphage f1 gene III protein (Haeuptle *et al.*, 1989), and 17 amino acids of its native transmembrane segment were sufficient to anchor the influenza A virus hemagglutinin (Doyle *et al.*, 1986). Transmembrane domains as short as 11 or 12 amino acids in length could interrupt translocation but were defective for integration into the membrane (Davis and Model, 1985; Davis *et al.*, 1985; Doyle *et al.*, 1986). Rather, defective anchor function of the tm segment may be related to the suboptimal hydrophobicity of its constituent amino acids. Hydrophobicity is a determinant of the ability of the segment to arrest translocation

in an aqueous channel and to effect disruption of the channel or a shift of the polypeptide into the lipid environment of the membrane, resulting in stable membrane integration (Walter and Lingappa, 1986; von Heijne, 1988; Simon and Blobel, 1991). If the consecutive 18 most hydrophobic residues (Gly-245 through Ile-262) are considered, the tm segment has an average hydrophobicity of 1.35, on a scale in which increasing positive values correlate with increasing hydrophobicity (Kyte and Doolittle, 1982). A functional transmembrane domain was predicted to have an average hydrophobicity of  $>1.6$ , using this same scale. The average hydrophobicity of the required entire C-terminus of the DEN4 prM, excluding Gln-263 and Arg-264 which are possibly extramembraneous, is 1.54.

We noted an enhancing effect of positive charge at or in the vicinity of conserved Arg-264, downstream from the tm segment, upon the capacity of the tm domain to effect stop-transfer and membrane integration in  $\Delta$ Ess mutants. This may be an indirect consequence of the demonstrated semicompetence of the tm segment for translocation arrest. Both the presence of charged residues and the conformation have been shown to reduce the translocatability or affect the membrane orientation of sequences adjacent to a transmembrane domain (for example, Boyd and Beckwith, 1990; Haeuptle *et al.*, 1989; Hartman *et al.*, 1989; Moreno *et al.*, 1980; Parks and Lamb, 1991; Sato *et al.*, 1990; von Heijne, 1989). Similarly, E sequences downstream from the tm segment in the  $\Delta$ Ess(2R) mutant may be relatively resistant to translocation compared to those in  $\Delta$ Ess(1R) and  $\Delta$ Essdr mutants, because of locally enhanced net charge or conformational differences related to the introduction of the extra arginine. Slowing or interdiction of translocation of these sequences might result in prolonged contact of the tm segment with the lipid environment of the membrane, enhancing the possibility for its membrane integration.

### E signal function

In these studies, we assayed for two manifestations of E signal sequence function, cleavage at the prM-E site and the reinitiation of the translocation of downstream E sequences. For the membrane-spanning fraction of  $\Delta$ Ess mutant chimeric molecules in the prM<sub>lumen</sub>-E<sub>cyto</sub> orientation, the intact tm segment apparently effected stop-transfer of the prM moiety. The mutated E signal peptide was available on the cytoplasmic side of the membrane to initiate translocation (and cleavage) of E, but it was completely defective. This indicated that hydrophobic amino acids deleted in  $\Delta$ Ess mutants (Fig. 1) were required for E signal function after stop-transfer of prM and confirmed the localization of E signal function to aa 265–279 for the DEN4 polyprotein.

Cleavage of a signal peptide normally occurs on the luminal side of the ER membrane (von Heijne, 1988); the

enzyme signal peptidase is an ER integral membrane protein oriented with its active site in the lumen (Blobel and Dobberstein, 1975; Lively and Walsh, 1983; Nicchita *et al.*, 1991). Signal recognition, mediated by signal recognition particle (SRP; Walter and Lingappa, 1986), results in the opening of an aqueous channel in the ER membrane (Simon and Blobel, 1991, 1992). After translocation is initiated, the N-terminus of the signal peptide remains on the cytoplasmic side of the ER membrane, while the C-terminus is inserted into the aqueous channel (Walter *et al.*, 1981). The remainder of the nascent translocating polypeptide enters the ER through the channel in the form of an enlarging hairpin loop held in place by the anchored signal peptide (Shaw *et al.*, 1988). Thus, the signalase cleavage site at the C-terminus of the signal peptide is presented for cleavage in the ER lumen.

We accounted for the defect in cleavage of membrane-spanning  $\Delta$ tm mutant molecules in the prM<sub>lumen</sub>-E<sub>cyto</sub> orientation by assuming that these proteins were anchored in the membrane by hydrophobic residues of the intact E signal peptide. Function of a signal as a transmembrane domain has been previously reported to occur if translocation has already been initiated by recognition of a signal in upstream sequences (Coleman *et al.*, 1985). As a result of aberrant insertion of the signal peptide into the membrane, the signalase cleavage site in membrane-spanning  $\Delta$ tm mutant prM+E molecules would reside in the cytoplasm, where it is inaccessible to signal peptidase. Thus translocation and cleavage of E in membrane-spanning chimeric  $\Delta$ tm mutant proteins was most likely preempted by function of the intact E signal as a transmembrane domain.

A small amount of signalase-cleaved prM was generated during processing of the  $\Delta$ tm mutant. It appeared to be integrated in membranes with a stability similar to that of membrane-spanning  $\Delta$ tm mutant prM+E molecules, and we postulate that it is derived from this population. Possibly, some unstably anchored chimeric molecules "slip" within the aqueous channel through which translocation is occurring, so that the signalase cleavage site becomes accessible to signal peptidase. Following cleavage, reintegration would have to occur. In support of this possibility, recent data demonstrate that under certain experimental conditions, traffic within the channel can be in both possible directions (Nicchita and Blobel, 1993).

For chimeric wholly intraluminal  $\Delta$ tm and  $\Delta$ Ess mutant polypeptides, we speculate that the cleavage defect was secondary to the defect in translocation arrest. Because translocation of prM was not arrested, the prM-E junction would be predicted to traverse the ER membrane in the N-terminus-first orientation, rather than in the usual C-terminus-first orientation (Walter *et al.*, 1981; Shaw *et al.*, 1988). Although the cleavage site presumably was accessible to signal peptidase, cleavage activity may have been sensitive to the N-to-C-terminal orientation of the target

amino acids during translocation. In support of this hypothesis, similar results were obtained in a previous study of cleavage of the influenza hemagglutinin signal peptide; the hemagglutinin signal also was not cleaved (and failed to arrest translocation) when it traversed the ER membrane N-terminus-first as part of an already translocating polypeptide (Finidori *et al.*, 1987).

### Processing of the flavivirus structural glycoproteins

These and previous data make it possible to propose a model for the processing of the flavivirus structural proteins, since the hydrophobic properties of the C-termini of prM and E are completely conserved in this genus (see Fig. 6; reviewed in Chambers *et al.*, 1990a).

#### (i) Processing of the nascent flavivirus polyprotein ap-

pears to begin with cotranslational recognition of the signal peptide at the C-prM junction (Markoff, 1989). Alternatively, cleavage of the capsid protein from the prM signal peptide (catalyzed by the virus-coded protease, NS3 [Chambers *et al.*, 1990c; Falgout *et al.*, 1991; Preugschat *et al.*, 1990; Wengler *et al.*, 1991]) may precede and facilitate prM signal recognition (Yamshchikov and Compans, 1993). The latter order of events seems especially likely to predominate late in infection, when NS3 is present in abundance. In either case, prM signal recognition is presumably mediated by interaction with SRP (Walter and Lingappa, 1986), and both alternative mechanisms ultimately result in the cytoplasmic disposition of C (not shown in Fig. 6). prM is likely to be extruded into the lumen of the ER through an aqueous channel as a hairpin loop, formed at the site of interaction of SRP with

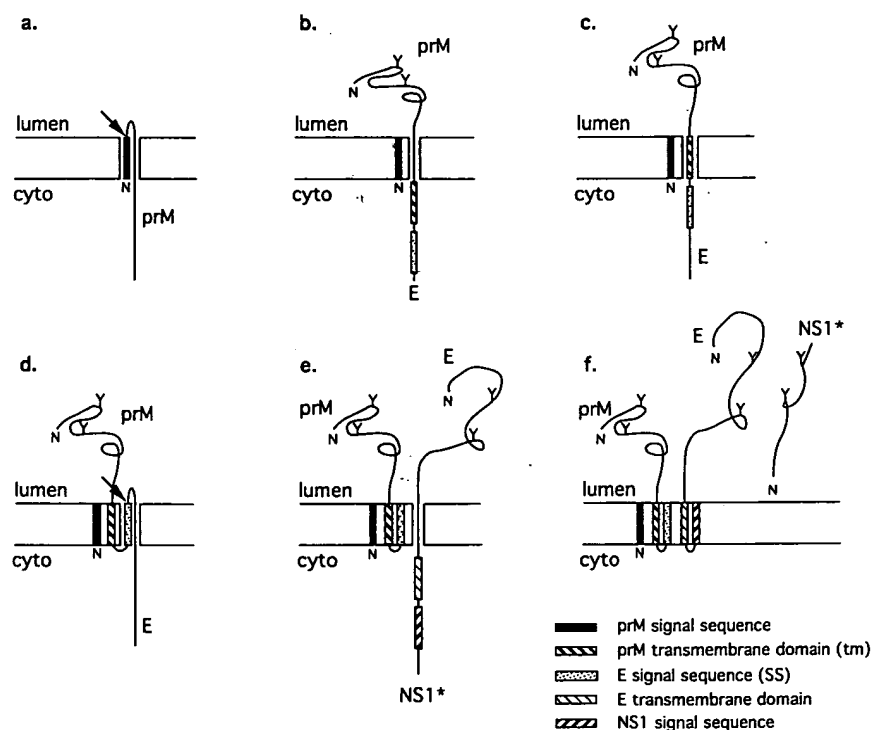


Fig. 6. Processing of the prM-E-NS1\* polypeptide encoded by pGWt DNA. The ER membrane is depicted, separating luminal (lumen) from cytoplasmic (cyto) spaces. (a) The prM signal sequence (aa 100–113 of the DEN4 polyprotein; Fig. 1) has been recognized, resulting in the opening of an aqueous channel such that the N-terminus (N) of the signal resides on the cytoplasmic side of the membrane. prM is shown translocating through the aqueous channel in the form of a hairpin loop. The diagonal arrow indicates the eventual site of cleavage of the prM signal from the N-terminus of processed prM by the membrane-anchored intraluminal enzyme signal peptidase. (b) Cleavage of the prM signal has occurred. The major ectodomain of prM has been translocated and diglycosylated (Y). The cleaved prM signal peptide is shown shifted into the lipid environment of the membrane, as suggested by others (for example, von Heijne, 1988). The N-terminus (N) of the mature prM is also indicated. (c) The transmembrane domain (tm) of prM (aa 243–263) transiently interrupts translocation of prM by coming into contact with the aqueous channel. Our data predict that this interaction is insufficient to arrest the translocation of all prM moieties and to mediate stable membrane integration. (d) The E signal sequence (SS; aa 265–279) has been recognized, stabilizing membrane integration of prM. We speculate that this requires the opening of a second aqueous channel, as shown. Nascent E is depicted translocating through this channel in the form of a hairpin loop. The conserved amphipathic domain in the carboxy-terminus of prM (aa 259–264) is disposed on the cytoplasmic side of the membrane. The diagonal arrow indicates the eventual site of cleavage of the E SS by signal peptidase. (e) Translocation of the major ectodomain of E is nearly complete. The C-terminus of prM is shown shifted into the lipid environment of the membrane. E is diglycosylated (Y). Cleavage of the E SS has occurred, freeing the N-terminus of processed E (N). (f) The transmembrane domain in E has transiently interrupted the translocation of E. Subsequent recognition of the NS1 signal has resulted in stable membrane integration of E, as well as translocation of NS1\* via a third aqueous channel. This results in signalase cleavage of the NS1 signal and glycosylation of NS1\*. NS1\* is depicted free in the lumen of the ER, since it lacks a candidate membrane anchor domain.

its receptor protein on the cytoplasmic side of the ER membrane (Walter *et al.*, 1981; Shaw *et al.*, 1988), as previously mentioned and depicted in Fig. 6a. The prM signal peptide, fixed in the membrane with its N-terminus on the cytoplasmic side would constitute the N-terminal end of the loop prior to its cleavage.

(ii) Cleavage of the prM signal by signal peptidase frees the N-terminus of prM within the ER lumen during translocation (Fig. 6b).

(iii) Translocation of prM is transiently interrupted by hydrophobic residues of the tm segment (Fig. 6c). As our results demonstrate, the tm segment is necessary but not sufficient for stable membrane anchoring of prM. Conserved Arg-264 may play a role in temporarily stabilizing prM in the membrane prior to E signal recognition, based upon the differing phenotypes of  $\Delta$ Ess(2R), (1R), and dr mutants.

(iv) Part or all of the amphipathic domain within the C-terminus of prM remains on the cytoplasmic side of the ER membrane as the E signal peptide is recognized and translocation of the polyprotein is reinitiated (Fig. 6d). The cytoplasmic localization of the amphipathic domain is suggested by the fact that  $\Delta$ R and  $\Delta$ QQR mutant prM were consistently found to be more stably membrane anchored (data not shown). This suggested that the amphipathic domain in wt prM does not participate in membrane anchoring. Recognition of the E signal is an essential step in the stable membrane anchoring of prM. We speculate that this step involves the opening of a second aqueous channel through which translocation of the polyprotein continues. Recognition of the E signal peptide may (Wessels and Speiss, 1988) or may not (Wickner and Lodish, 1985) require SRP binding of the E signal.

(v) Cleavage of the E signal peptide defines the C-terminus of prM. Thus, prM is "hooked" into the membrane such that the tm segment and the E signal, respectively, form the proximal and distal intramembraneous arms of the hook (Fig. 6e). Translocation of E sequences in the polyprotein into the ER lumen proceeds via the second aqueous channel afforded by E signal peptide recognition.

(vi) Interruption of the translocation of E, its membrane anchoring, and cleavage of the E-NS1 site are effected by analogous processes (Fig. 6f).

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## REFERENCES

- BAUSE, E., and LEGLER, G. (1981). The role of the hydroxy amino acid in the triplet sequence Asn-Xaa-Thr (Ser) for the N-glycosylation step during glycoprotein biosynthesis. *Biochem. J.* 195, 639-644.
- BLOBEL, G. (1980). Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. USA* 77, 1496-1500.
- BLOBEL, G., and DOBBERSTEIN, B. (1975). Transfer of proteins across membranes II. Reconstitution of functional rough microsomes from heterologous components. *J. Cell Biol.* 67, 852-862.
- BOYD, D., and BECKWITH, J. (1990). The role of charged amino acids in the localization of secreted and membrane proteins. *Cell* 62, 1031-1033.
- CHAMBERS, T. J., HAHN, C. S., GALLER, R., and RICE, C. M. (1990a). Flavivirus genome organization, expression, and replication. *Annu. Rev. Microbiol.* 44, 649-688.
- CHAMBERS, T. J., MCCOURT, D. W., and RICE, C. M. (1990b). Production of yellow fever virus proteins in infected cells: identification of discrete polyprotein species and analysis of cleavage kinetics using region-specific polyclonal antisera. *Virology* 177, 159-174.
- CHAMBERS, T. J., WEIR, R. C., GRAKOUI, A., MCCOURT, D. W., BAZAN, J. F., FLETTERICK, R. J., and RICE, C. M. (1990c). Evidence that the N-terminal domain of nonstructural protein NS3 from yellow fever virus is a serine protease responsible for site-specific cleavages in the viral polyprotein. *Proc. Natl. Acad. Sci. USA* 87, 8898-8902.
- COLEMAN, J. M., INUKAI, M., and INOUE, M. (1985). Dual functions of the signal peptide in protein transfer across the membrane. *Cell* 43, 351-360.
- DAVIS, N. G., BOEKE, J. D., and MODEL, P. (1985). Fine structure of a membrane anchor domain. *J. Mol. Biol.* 181, 111-121.
- DAVIS, N. G., and MODEL, P. (1985). An artificial anchor domain: hydrophobicity suffices to stop transfer. *Cell* 41, 607-614.
- DESPRÉS, P., RUIZ-LINARÉS, A., CAHOUR, A., GIRARD, M., WYCHOWSKI, C., and BOULOY, M. (1990). The 15 amino acid residues preceding the amino terminus of the envelope protein in the yellow fever virus polyprotein precursor act as a signal peptide. *Virus Res.* 16, 59-76.
- DOYLE, C., SAMBROOK, J., and GETHING, M. J. (1986). Analysis of progressive deletions of the transmembrane and cytoplasmic domains of influenza hemagglutinin. *J. Cell Biol.* 103, 1193-1203.
- EISENBERG, D. (1984). Three-dimensional structure of membrane and surface proteins. *Annu. Rev. Biochem.* 53, 595-623.
- FALGOUT, B., PETHEL, M., ZHANG, Y.-M., and LAI, C.-J. (1991). Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. *J. Virol.* 65, 2467-2475.
- FINIDORI, J., RIZZOLO, L., GONZALES, A., KREIBACH, G., ADESNIK, M., and SABATINI, D. D. (1987). The influenza HA insertion signal is not cleaved and does not halt translocation when presented to the ER membrane as part of a translocating polypeptide. *J. Cell Biol.* 104, 1705-1714.
- FUJIKI, Y., HUBBARD, A. L., FOWLER, S., and LAZAROW, P. B. (1982). Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 93, 97-102.
- HAEUPTLE, M.-T., FLINT, N., GOUGH, N. M., and DOBBERSTEIN, B. (1989). A tripartite structure of the signals that determine protein insertion into the endoplasmic reticulum membrane. *J. Cell Biol.* 108, 1227-1236.
- HARTMANN, E., RAPOPORT, T. A., and LODISH, H. F. (1989). Predicting the orientation of eukaryotic membrane-spanning proteins. *Proc. Natl. Acad. Sci. USA* 86, 5786-5790.
- HASE, T., SUMMERS, P. L., ECKELS, K. H., and BAZE, W. B. (1987a). Maturation process of Japanese encephalitis virus in cultured mosquito cells in vitro and mouse brain cells in vivo. *Arch. Virol.* 96, 135-151.
- HASE, T., SUMMERS, P. L., ECKELS, K. H., and BAZE, W. B. (1987b). An electron and immunoelectron microscopic study of dengue-2 virus infection of cultured mosquito cells: maturation events. *Arch. Virol.* 92, 273-291.
- KOZAK, M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* 44, 283-292.
- KYTE, J., and DOOLITTLE, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157, 105-132.

- LEARY, K., and BLAIR, C. D. (1980). Sequential events in the morphogenesis of Japanese encephalitis virus. *J. Ultrastruct. Res.* **72**, 123-129.
- LIVELY, M. O., and WALSH, K. A. (1983). Hen oviduct signal peptidase is an integral membrane protein. *J. Biol. Chem.* **258**, 9488-9495.
- MACKOW, E., MAKINO, Y., ZHAO, B., ZHANG, Y.-M., MARKOFF, L., BUCKLER-WHITE, A., GUILER, M., CHANOCK, R., and LAI, C.-J. (1987). The nucleotide sequence of dengue type 4 virus: analysis of genes coding for non-structural proteins. *Virology* **159**, 217-228.
- MARKOFF, L. (1989). In vitro processing of dengue virus structural proteins, cleavage of the pre-membrane protein. *J. Virol.* **63**, 3345-3352.
- MORENO, F., FOWLÉR, A. V., HALL, M., SILHAVY, T. J., ZABIN, I., and SCHWARTZ, M. (1980). A signal sequence is not sufficient to lead  $\beta$ -galactosidase out of the cytoplasm. *Nature* **286**, 356-359.
- NICCHITA, C. V., and BLOBEL, G. (1993). Lumenal proteins of the mammalian endoplasmic reticulum are required to complete protein translocation. *Cell* **73**, 989-998.
- NICCHITA, C. V., MIGLIACCIO, G., and BLOBEL, G. (1991). Biochemical fractionation and assembly of the membrane components that mediate nascent chain targeting and translocation. *Cell* **65**, 587-598.
- NOWAK, T., FÄRBER, P. M., WENGLER, G., and WENGLER, G. (1989). Analyses of the terminal sequences of West Nile virus structural proteins and of the *in vitro* translation of these proteins allow the proposal of a complete scheme of the proteolytic cleavages involved in their synthesis. *Virology* **169**, 365-376.
- NOWAK, T., and WENGLER, G. (1987). Analysis of disulfides present in the membrane proteins of the West Nile flavivirus. *Virology* **156**, 127-137.
- PARKS, G. D., and LAMB, R. A. (1991). Topology of eukaryotic type II membrane proteins: importance of N-terminal positively charged residues flanking the hydrophobic domain. *Cell* **64**, 777-787.
- PREUGSCHAT, F., YAO, C.-W., and STRAUSS, J. H. (1990). In vitro processing of dengue virus type 2 nonstructural proteins NS2A, NS2B, and NS3. *J. Virol.* **64**, 4364-4374.
- RUIZ-LINARES, A., CAHOUR, A., DESPRES, A., GIRARD, P., and BOULOY, M. (1989). Processing of yellow fever virus polyprotein, role of cellular proteases in maturation of the structural proteins. *J. Virol.* **63**, 4199-4209.
- SATO, T., SAKAGUCHI, M., MIHARA, K., and OMURA, T. (1990). The amino-terminal structures that determine topological orientation of cytochrome P-450 in microsomal membrane. *EMBO J.* **9**, 2391-2397.
- SCHÄGGER, H., and VON JAGOW, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal. Biochem.* **166**, 368-379.
- SHAW, A. S., ROTTIER, P. J. M., and ROSE, J. K. (1988). Evidence for the loop model of signal-sequence insertion into the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA* **85**, 7592-7596.
- SIMON, S. M., and BLOBEL, G. (1991). A protein-conducting channel in the endoplasmic reticulum. *Cell* **65**, 371-380.
- SIMON, S. M., and BLOBEL, G. (1992). Signal peptides open protein-conducting channels in *E. coli*. *Cell* **69**, 677-684.
- VON HEIJNE, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14**, 4683-4690.
- VON HEIJNE, G. (1989). Control of topology and mode of assembly of a polytopic membrane protein by positively charged residues. *Nature* **341**, 456-458.
- VON HEIJNE, G. (1988). Transcending the impenetrable: how proteins come to terms with membranes. *Biochim. Biophys. Acta* **947**, 307-333.
- WALTER, P., IBRAHIMI, I., and BLOBEL, G. (1981). Translocation of proteins across the endoplasmic reticulum I. signal recognition protein (SRP) binds to in-vitro-assembled polysomes synthesizing secretory protein. *J. Cell Biol.* **91**, 545-550.
- WALTER, P., and LINGAPPA, V. R. (1986). Mechanism of protein translocation across the endoplasmic reticulum membrane. *Annu. Rev. Cell Biol.* **2**, 499-516.
- WENGLER, G., CZAYA, G., FÄRBER, P. M., and HEGEMAN, J. H. (1991). In vitro synthesis of West Nile virus proteins indicates that the amino-terminal segment of the NS3 protein contains the active centre of the protease which cleaves the viral polyprotein after multiple basic amino acids. *J. Gen. Virol.* **72**, 851-858.
- WESSELS, H. P., and SPIESS, M. (1988). Insertion of a multi spanning membrane protein occurs sequentially and requires only one signal sequence. *Cell* **55**, 61-70.
- WICKNER, W. T., and LODISH, H. F. (1985). Multiple mechanisms of protein insertion into and across membranes. *Science* **230**, 400-406.
- YAMSHCHIKOV, V. F., and COMPANS, R. W. (1993). Regulation of the late events in flavivirus protein processing and maturation. *Virology* **192**, 38-51.
- ZHAO, B., MACKOW, E., BUCKLER-WHITE, A., MARKOFF, L., CHANOCK, R. M., LAI, C.-J., and MAKINO, Y. (1986). Cloning full-length dengue type 4 viral DNA sequences: analysis of genes coding for structural proteins. *Virology* **155**, 77-88.
- ZOLLER, M. J., and SMITH, M. (1983). Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13-derived vectors. *Methods Enzymol.* **100**, 468-500.

## Comparison of Protective Immunity Elicited by Recombinant Vaccinia Viruses That Synthesize E or NS1 of Japanese Encephalitis Virus

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Immunization with recombinant vaccinia viruses that specified the synthesis of Japanese encephalitis virus (JEV) glycoproteins protected mice from a lethal intraperitoneal challenge with JEV. Recombinants which coexpressed the genes for the structural glycoproteins, prM and E, elicited high levels of neutralizing (NEUT) and hemagglutination inhibiting (HAI) antibodies in mice and protected mice from a lethal challenge by JEV. Recombinants expressing only the gene for the nonstructural glycoprotein, NS1, induced antibodies to NS1 but provided low levels of protection from a similar challenge dose of JEV. Antibodies to the NS3 protein in postchallenge sera, representing the degree of infection with challenge virus, were inversely correlated to NEUT and HAI titers and levels of protection. These results indicate that although vaccinia recombinants expressing NS1 can provide some protection from lethal JEV infection, recombinants expressing prM and E elicited higher levels of protective immunity. © 1991 Academic Press, Inc.

### INTRODUCTION

Japanese encephalitis virus (JEV) is one of the most important human and animal pathogens of the family *Flaviviridae* (Shope, 1980; Monath, 1986). Although a highly effective inactivated vaccine for JEV has been developed, it is expensive and similar vaccines have not been obtainable for other flaviviruses, notably dengue. Since nucleotide sequence analyses have revealed that flaviviruses are very similar at the molecular level (Chambers *et al.*, 1990), and since several animal models exist for JEV, we have pursued the production of recombinant vaccines against JEV as a method for learning about flavivirus vaccines in general.

Flavivirus proteins are encoded by a single long translational open reading frame (ORF) present in the positive-strand genomic RNA. The flavivirus genome encodes a capsid protein, C, a membrane protein, M (which is found in infected cells as the precursor prM), an envelope glycoprotein, E, and at least seven nonstructural proteins, including the nonstructural glycoprotein, NS1 (Chambers *et al.*, 1990).

Passively administered monoclonal antibodies (MAbs) specific for each of the three flavivirus glycoproteins (prM, E, and NS1) are capable of providing protection from lethal infection by the homologous virus (Heinz *et al.*, 1983; Mathews and Roehrig, 1984; Schlesinger *et al.*, 1985; Gould *et al.*, 1986; Kaufman *et al.*, 1987, 1989; Henchal *et al.*, 1988; Kimura-Kuroda and Yasui, 1988; Mason *et al.*, 1989). In the case of MAbs to E, passive protection correlated with *in vitro* neutralizing activity, but nonneutralizing antibodies specific for prM and NS1 were also identified. Work by Schlesinger and co-workers provided additional evidence that NS1 immunity could protect animals from infection by demonstrating that purified NS1 was protective (Schlesinger *et al.*, 1985, 1986, 1987). Furthermore, since antibodies to structural proteins of one dengue serotype may "enhance" infection by other serotypes, an NS1-based subunit or recombinant vaccine may be desirable (Halstead, 1988).

Several different recombinant DNA strategies have been employed to generate candidate flavivirus vaccines. These strategies have included purified *Escherichia coli* fusion proteins (Cane and Gould, 1988; Mason *et al.*, 1989), crude lysates from moth cells infected with recombinant baculoviruses (Zhang *et al.*, 1988; Matsuura *et al.*, 1989; McCown *et al.*, 1990; Deubel *et al.*, 1991), and live recombinant vaccinia viruses (Deubel *et al.*, 1988; Zhao *et al.*, 1987; Haishi *et al.*, 1989; Bray *et al.*, 1989; Falgout *et al.*, 1990; Hahn *et al.*, 1990; Putnak and Schlesinger, 1990; Yasuda *et al.*, 1990; Mason *et al.*, 1991; Men *et al.*, 1991). Several of these, notably the recombinant baculovirus and vaccinia strategies, have yielded promising protection data.

We have previously described recombinant vaccinia viruses expressing portions of the JEV ORF extending

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from prM to NS2B. One of these viruses (vP555), which correctly expressed the prM, E, and NS1 proteins, induced the synthesis of extracellular particles that behaved like empty viral envelopes and protected mice from a lethal JEV challenge (Mason *et al.*, 1991). In the current study we have defined the JEV genes needed to produce extracellular particles and we have compared the protective capacity of a recombinant virus that produces these particles to viruses that specify the synthesis of NS1 alone.

## MATERIALS AND METHODS

### Cell lines and virus strains

A thymidine kinase mutant of the Copenhagen strain of vaccinia virus, vP410 (Guo *et al.*, 1989), was used to generate recombinants vP825, vP829, vP857, and vP864 (see below). The generation of vP555 has been previously described (Mason *et al.*, 1991). All vaccinia virus stocks were produced in VERO (ATCC CCL81) cells in Eagle's minimal essential medium plus 10% heat-inactivated fetal bovine serum (FBS). Biosynthetic studies were performed using VERO cells grown at 37° in MEM supplemented with 5% FBS and antibiotics, or HeLa (ATCC CCL2) cells grown under the same conditions except using 10% FBS and nonessential amino acids. The Nakayama strain of JEV used in all *in vitro* experiments has been described (Mason, 1989). Animal challenge experiments were performed using the highly pathogenic Beijing P3 strain of JEV (multiple mouse passage; Huang, 1982).

### Preparation of JEV cDNA encoding the C protein

cDNA encoding the C protein of JEV was obtained by a modification of the method of Okayama and Berg (1982) using Moloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD) (D'Alessio and Gerard, 1988). Genomic RNA was isolated from virions prepared from suspension cultures of C6/36 cells (Igarashi, 1978) infected with the Nakayama strain of JEV. First strand cDNA synthesis was primed from a synthetic oligonucleotide complementary to bases 104 to 123 of the E coding region of JEV (McAda *et al.*, 1987). The double-stranded cDNA was ligated to synthetic oligonucleotides containing the *EcoRI* site (New England Biolabs, Beverly, MA), inserted into phosphatase-treated, *EcoRI*-cleaved pBR322 (New England Biolabs), and the resulting DNA was used to transform *E. coli* strain DH5 cells (BRL). Plasmids were analyzed by restriction enzyme digestion and a plasmid (pC20) containing cDNA corresponding to 81 nucleotides of noncoding RNA and the C and prM coding regions was identified. pC20 was digested with *EcoRI* and at an

internal *DraI* site situated 28 bp 5' of the ATG initiation codon and the resulting fragment containing the C and prM coding regions was inserted into *SmaI*-*EcoRI* digested pUC18, creating plasmid, pDr20. The sequence of the C coding region of pC20, combined with an updated sequence of the prM, E, NS1, NS2A, and NS2B coding regions of the Nakayama strain of JEV, has been deposited with GenBank; all nucleotide coordinates listed in this publication are based on this updated sequence, with numbering beginning at the C protein methionine (Met) initiation codon.

### Cloning of JEV genes into a vaccinia virus donor plasmid

Restriction enzymes and T4 DNA ligase were obtained from BRL, New England Biolabs, or Boehringer-Mannheim Biochemicals (Indianapolis, IN). Standard recombinant DNA techniques were used (Maniatis *et al.*, 1982) with minor modifications for cloning, screening, and plasmid purification. Nucleic acid sequences were confirmed using standard dideoxy chain-termination reactions (Sanger *et al.*, 1977) on alkaline-denatured double-stranded plasmid templates. Oligonucleotides were synthesized using standard chemistries (Biosearch 8700, San Rafael, CA; Applied Biosystems 380B, Foster City, CA). Plasmids containing cDNA encoding prM, E, NS1, NS2A, and NS2B from the Nakayama strain of JEV have been described (McAda *et al.*, 1987; Mason *et al.*, 1991).

Plasmid pDr20, containing JEV cDNA (nucleotides -28 to 1000; see above), was digested with *BamHI* and *EcoRI* and the JEV cDNA insert was cloned into pIBI25 (IBI, New Haven, CT) generating plasmid pJEV18. pJEV18 was digested with *Apal* within the JEV sequence (nucleotide 23) and *XhoI* within pIBI25 and ligated to annealed oligonucleotides J90 and J91 (Fig. 1; containing an *XhoI* sticky end, *SmaI* site, and JEV nucleotides 1 to 23) generating plasmid pJEV19. pJEV19 was digested with *XhoI* within pIBI25 and *AccI* within JEV sequences (nucleotide 602) and the resulting 613-bp fragment was cloned into the *XhoI* and *AccI* fragment of pJEV2 (Mason *et al.*, 1991), generating plasmid pJEV20 (containing JEV sequences from the ATG of C through the *SacI* site (nucleotide 2124) found in the last third of E).

The *SmaI*-*SacI* fragment from pJEV8 (a plasmid analogous to pJEV1 (Mason *et al.*, 1991) in which TTCTTGT nucleotides 1304 to 1310 was changed to TCTTTGT) containing JEV sequences from the last third of E through the first two amino acids (aa) of NS2B (nucleotides 2124 to 4126), the plasmid origin, and vaccinia sequences was ligated to the purified *SmaI*-*SacI* insert from pJEV20 yielding pJEV22-1. The 6 bp correspond-



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J90 5'-TCGAG CCCGGG atg ACTAAAAACACGGA GGGCC-3'
J91 3'-C GGGCCC TAC TGATTTTTTGGTCT C -5'
           XhoI   SmaI                               ApaI

J94 5'-C T tga IIIIII tga CGGCCG A -3'
J95 3'-GTACG A ACT AAAATA ACT GCGGCG TTCGA-5'
           SphI                               EagI, HindIII

J96+J97 5'-GGG atg GGCCTTACGCACGAGACCGATCAATTGCTTTGGCCTTCTTAGCCACAGGAGGTGTGCTCGTGTCTTAGCGACCAATGT GCATG-3'
J99+J98 3'-CCC TAC CCGCAATTGCGTGTCTGGCTAGTTACGAAACCGGAGAGATCGGTGCTCTCCACAGCAGCACAAGATCGCTGTTACA C -5'
                                           SphI

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FIG. 1. Oligonucleotides used to construct the vaccinia virus donor plasmids; the translation stop (tga) and start (atg) sites are lower case and the early transcription stop (TTTTTAT) is underlined.

ing to the unique *SmaI* site used to construct pJEV22-1 were removed using oligonucleotide-directed double-strand break mutagenesis (Mandecki, 1986) creating pJEV24 in which the H6 early/late promoter immediately preceded the ATG start codon.

Plasmid pJEV7 (Mason *et al.*, 1991) was digested with *SphI* within JEV sequences (nucleotide 2380) and *HindIII* within pBI24 and ligated to annealed oligonucleotides J94 and J95 (Fig. 1; containing a *SphI* sticky end, translation stop, a vaccinia early transcription termination signal (TTTTTAT; Yuen and Moss, 1987), a second translation stop, and *EagI* site and a *HindIII* sticky end) to generate plasmid pJEV25 which contains JEV cDNA extending from the *SacI* site (nucleotide 2124) in the last third of E through the C-terminus of E. The *SacI*-*EagI* fragment from pJEV25 was ligated to the *SacI*-*EagI* fragment of pJEV8 (containing JEV cDNA encoding the prM signal sequence, prM, and the N-terminal two thirds of E (nucleotides 337 to 2124), and the plasmid origin and vaccinia sequences) yielding pJEV26. A unique *SmaI* site preceding the ATG start codon was removed as described above, creating pJEV27 in which the H6 promoter immediately preceded the ATG start codon.

Oligonucleotides J96, J97, J98, and J99 (Fig. 1; containing JEV nucleotides 2293 to 2380 with the *SphI* sticky end) were annealed and ligated to *SmaI*-*SphI* digested pBI25 generating pJEV28. pJEV28 was digested with *HpaI* within the JEV sequence (nucleotide 2301) and with *HindIII* within the pBI25 sequence and ligated to the *HpaI*-*HindIII* fragment from pJEV1 or *HpaI*-*HindIII* fragment from pJEV7 (Mason *et al.*, 1991) yielding pJEV29 (containing a *SmaI* site followed by JEV cDNA encoding the NS1 signal sequence, NS1, NS2A (nucleotides 2293 to 4126) or pJEV30 (containing a *SmaI* site followed by JEV cDNA encoding the NS1 signal sequence, NS1, NS2A, NS2B (nucleotides 2293 to 4512)).

The *SmaI*-*EagI* fragment from pJEV29 was ligated to *SmaI*-*EagI* digested pTP15 (Mason *et al.*, 1991) yielding pJEV31. The 6 bp corresponding to the unique *SmaI* site used to produce pJEV31 were removed as

described above creating pJEV33 in which the H6 promoter immediately preceded the ATG start codon.

The *SmaI*-*EagI* fragment from pJEV30 was ligated to *SmaI*-*EagI* digested pTP15 yielding pJEV32. The 6 bp corresponding to the unique *SmaI* site used to produce pJEV32 were removed as described above creating pJEV34 in which the H6 promoter immediately preceded the ATG start codon.

### Construction of vaccinia virus recombinants

Procedures for transfection of recombinant donor plasmids into tissue culture cells infected with a rescuing vaccinia virus and identification of recombinants by *in situ* hybridization on nitrocellulose filters have been described (Panicali and Paoletti 1982; Guo *et al.*, 1989). pJEV24, pJEV27, pJEV33, and pJEV34 were transfected into vP410-infected cells to generate the vaccinia recombinants vP825, vP829, vP857, and vP864, respectively (Fig. 2).

### In vitro virus infection and radiolabeling

HeLa cell monolayers prepared in 35-mm-diameter dishes were infected with vaccinia viruses (m.o.i. of 2) or JEV (m.o.i. of 5). At 16 hr (vaccinia) or 40 hr (JEV) postinfection, the cells were pulse labeled for 2 hr with medium containing [<sup>35</sup>S]Met and chased for 6 hr in the presence of excess unlabeled Met as described by Mason *et al.* (1991). Radioactive JEV antigens used to check pre- and postchallenge mouse sera (see below) were prepared by pulse labeling JEV-infected VERO cells for 3 hr at 24 hr postinfection.

### Radioimmunoprecipitation and polyacrylamide gel electrophoresis

Radiolabeled cell lysates and culture fluids were prepared and the viral proteins were immunoprecipitated and resolved by SDS-containing polyacrylamide gel electrophoresis (SDS-PAGE) as described by Mason *et al.* (1991).

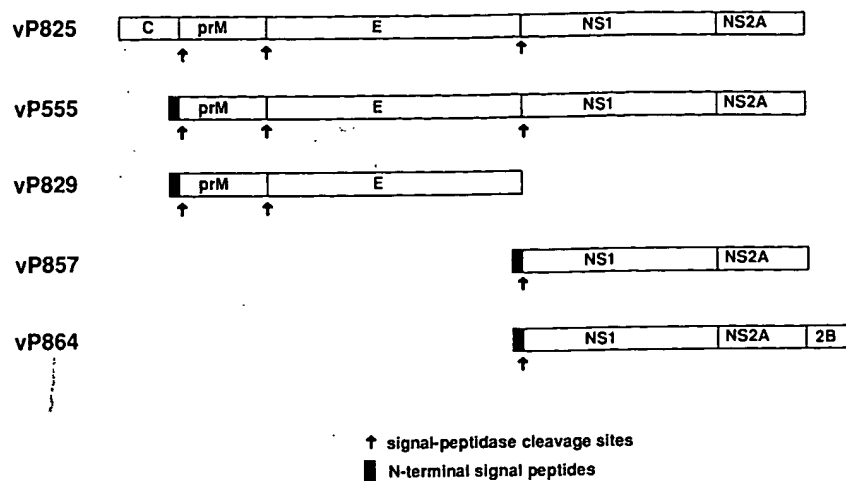


FIG. 2. Map of the JEV coding regions inserted in the recombinant vaccinia viruses.

### Animal protection experiments

Mouse protection experiments were performed as previously described (Mason *et al.*, 1991). Briefly, groups of 20 3-week-old outbred Swiss mice were immunized by intraperitoneal (i.p.) injection with  $10^7$  pfu of vaccinia virus, and 3 weeks later sera were collected from selected mice. One half of the mice in each group were then either reinoculated with the recombinant virus or challenged by i.p. injection with the P3 strain of JEV. Three weeks later, the boosted animals were bled and then challenged with the P3 strain of JEV. Following challenge, mice were observed at daily intervals for 3 weeks and lethal-dose titrations were performed in each challenge experiment using littermates of the experimental animals. In addition, sera were collected from all surviving animals 4 weeks after challenge.

### Evaluation of immune responses

Pooled sera were tested for their ability to precipitate JEV proteins from detergent-treated cell lysates or culture fluids obtained from [ $^{35}$ S]Met-labeled JEV-infected cells as described by Mason *et al.* (1991). Hemagglutination inhibition (HAI) tests and neutralization (NEUT) tests were performed essentially as described by Mason *et al.* (1991).

## RESULTS

### Structures of the recombinant vaccinia viruses

Four new vaccinia recombinants were constructed that contained portions of the JEV coding region extending from C through NS2B inserted in the HA locus. The JEV cDNA sequences contained in these recombinant viruses, along with vP555 (Mason *et al.*, 1991), are shown in Fig. 2. In all recombinant viruses the

sense strand of the JEV cDNA was positioned behind the early/late H6 promoter present in the vP410 parent strain of vaccinia, and translation was expected to be initiated from naturally occurring JEV Met codons located at the 5' ends of the viral cDNA sequences (Fig. 2).

Recombinant vP825 encodes the capsid protein C, structural protein precursor prM, the structural glycoprotein E, the nonstructural glycoprotein NS1, and the nonstructural protein NS2A. Recombinant vP555 (Mason *et al.*, 1991) encodes the putative 15 aa signal sequence preceding the N-terminus of prM, prM, E, NS1, and NS2A. Recombinant vP829 encodes the putative signal sequence of prM, prM, and E. Recombinant vP857 contains a cDNA encoding the predicted 30 aa signal sequence for NS1 followed by NS1 and NS2A. Recombinant vP864 contains a cDNA encoding the same proteins as vP857 with the addition of NS2B. In recombinants vP825 and vP829 a potential vaccinia virus early transcription termination signal in E (TTTTGT; nucleotides 1304–1310) was modified to TCTTGT without altering the aa sequence. This change was made in an attempt to increase the level of expression of E since this sequence has been shown to increase transcription termination in *in vitro* transcription assays (Yuen and Moss, 1987).

### E was properly processed when expressed by recombinant vaccinia viruses

The data from the pulse-chase experiments depicted in Fig. 3 demonstrate that proteins identical in size to E were synthesized in cells infected with all recombinant vaccinia viruses containing the E gene. In the case of cells infected with JEV, vP555, and vP829,

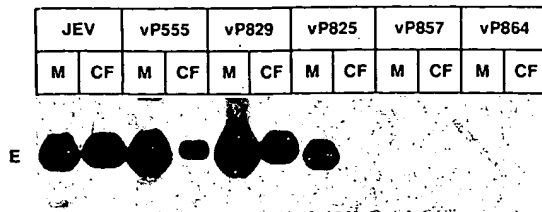


Fig. 3. Comparison of the E protein produced by JEV infection or infection with the recombinant vaccinia viruses. HeLa cells were infected with JEV or recombinant vaccinia viruses, labeled for 2 hr with [ $^{35}$ S]Met, and chased for 6 hr. Equal portions of the cell monolayer (M) or culture fluid (CF) prepared from each cell layer were immunoprecipitated with a MAb specific for E and then subjected to SDS-PAGE analysis.

an E protein that migrated slower in SDS-PAGE was also detected in the culture fluid harvested from the infected cells (Fig. 3). We have previously shown that this extracellular form of E produced by JEV- and vP555-infected cells contains mature N-linked glycans (Mason, 1989; Mason *et al.*, 1991), and we have confirmed this finding by endoglycosidase treatment of the extracellular forms of E harvested from vP829-infected cells (results not shown).

Interestingly, vP825, which contains the C coding region in addition to prM and E specifies the synthesis of E in a form that is not released into the extracellular fluid (see Figs. 2 and 3). In addition, vP829-infected cells released more E protein than vP555-infected cells. Immunoprecipitates prepared from radiolabeled vaccinia-infected cells using a MAb specific for M (and prM) revealed that prM was synthesized in cells infected with vP555, vP825, and vP829 (data not shown).

The extracellular fluid harvested from cells infected with vP555 and vP829 contained an HA activity that was undetectable in the culture fluid of cells infected with vP410, vP825, vP857, and vP864. The HA activity observed in the culture fluid of vP829-infected cells was eight times as high as that obtained from vP555-infected cells. This HA appeared similar to the HA produced in JEV-infected cells based on its inhibition by anti-JEV antibodies and its pH optimum (see Mason *et al.*, 1991). Analysis of sucrose density gradients prepared with culture fluids obtained from infected cells identified a peak of HA activity in the vP829 sample that comigrated with the peak of slowly sedimented hemagglutinin (SHA) found in the JEV culture fluids (data not shown). This result indicated that vP829-infected cells produced extracellular particles similar to the empty viral envelopes containing E and M which we observed in the culture fluids harvested from vP555-infected cells (Mason *et al.*, 1991).

### NS1 was properly processed when expressed by recombinant vaccinia viruses

The data from the pulse-chase experiments depicted in Fig. 4 demonstrate that a protein identical in size to authentic NS1 was synthesized in cells infected with vP555, vP825, vP857, or vP864. In addition, these cells produced a higher molecular weight form of NS1, NS1', which we have previously shown is produced by alternative processing of the sequences encoded by the NS2A region of the JEV genome (Mason *et al.*, 1987). Consistent with our previous experiments, we found that NS1 produced by vP555-infected cells was released into the culture fluid of infected cells in a higher molecular weight form. NS1 was also released into the culture fluid of cells infected with vP857 and vP864. Comparison of the synthesis of NS1 from vaccinia viruses containing either the NS2A (vP857) or both the NS2A and NS2B (vP864) coding regions showed that the presence or absence of the NS2B coding region had no effect on NS1 expression, consistent with previous data showing that only the NS2A gene is needed for the proper processing of NS1 (Falgout *et al.*, 1989; Mason *et al.*, 1991).

Examination of the autoradiogram shown in Fig. 4 suggests that cells infected with vP825 did not release NS1. Although overexposure of this and other autoradiograms did show that some NS1 was released from vP825-infected cells, the efficiency of release was more than 10 times less than that for NS1 synthesized in vP555-, vP857-, or vP864-infected cells.

### Recombinant vaccinia viruses induced immune responses to JEV antigens

Prechallenge sera pooled from selected animals in each group were tested for their ability to immunoprecipitate radiolabeled E and NS1. The results of these

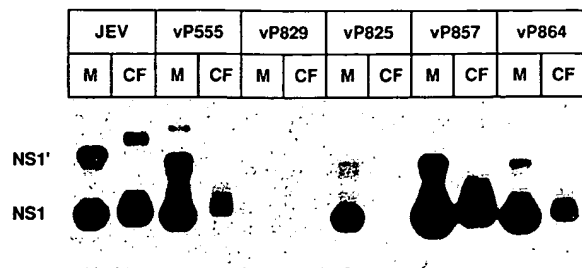


Fig. 4. Comparison of the NS1 proteins produced by JEV infection or infection with the recombinant vaccinia viruses. HeLa cells were infected with JEV or recombinant vaccinia viruses, labeled for 2 hr with [ $^{35}$ S]Met, and chased for 6 hr. Equal fractions of the cell monolayer (M) or culture fluid (CF) prepared from each cell layer were immunoprecipitated with a MAb specific for NS1 and then subjected to SDS-PAGE analysis.

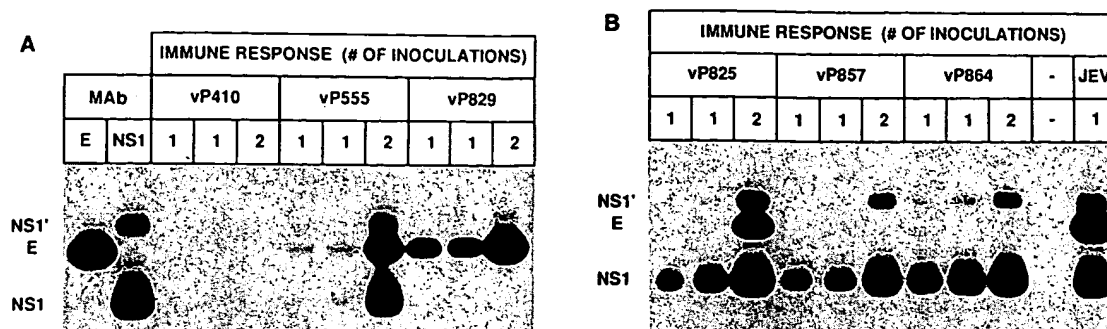


FIG. 5. Analysis of the JEV-specific reactivity of prechallenge sera from mice vaccinated with the recombinant vaccinia viruses or the parental vaccinia virus, vP410. Sera collected from a subset of the animals used in the protection experiments (see Tables 1 and 2) were pooled and aliquots were tested for their ability to immunoprecipitate radiolabeled proteins harvested from the culture fluid of JEV-infected cells. (A) The positions of the E and NS1 proteins precipitated with MAbs and the radioimmunoprecipitations obtained from the pooled sera from the two groups of animals vaccinated once (1) or twice (2) with vP410, vP555, or vP829. (B) The reactivity of sera obtained from groups of animals vaccinated with vP825, vP857, or vP864; the two lanes on the right side of the autoradiogram in Panel B were prepared from samples immunoprecipitated with sera obtained from uninoculated mice (-) or from a mouse that survived a normally lethal dose of JEV (JEV).

studies (Fig. 5) demonstrated: (1) the following order of immune response to E, vP829 > vP555 > vP825, (2) all viruses encoding NS1 and NS2A induced antibodies to NS1, and (3) all immune responses were increased by a second inoculation with the recombinant viruses. Analysis of the neutralization and HAI data for the sera collected from these animals confirmed the results of the immunoprecipitation analyses, showing that the immune response to E as demonstrated by radioimmunoprecipitation correlated well with these other serological tests (Table 1).

#### Vaccination with the recombinant viruses provided protection from lethal JEV infection

All of the recombinant vaccinia viruses were able to provide mice with some protection from lethal JEV infection by the peripherally pathogenic P3 strain of JEV (Huang, 1982) (Table 2). These studies confirmed the protective potential of vP555 (Mason *et al.*, 1991) and demonstrated similar protection in animals inoculated with vP825 and vP829. Recombinant viruses vP857 and vP864 which induced strong immune responses to NS1 (see above, Fig. 5) showed much lower levels of protection, but mice inoculated two times with these recombinants were still significantly protected when compared to mice inoculated with the control virus, vP410 ( $P < 0.05$  by the  $\chi^2$  test; Table 2).

#### Postchallenge immune responses document the level of JEV replication

In order to obtain a better understanding of the mechanism of protection from lethal challenge in vaccinated animals, the ability of antibodies in postchallenge sera to recognize JEV antigens was evaluated. Since the

nonstructural protein, NS3, was not expressed in any of the recombinant viruses and since it induces high levels of antibodies in hyperimmunized mice (Mason *et al.*, 1987), reactivity to NS3 was chosen as an indicator of viral replication in the sera of animals surviving infection. The reactivity of postchallenge sera with NS3 correlates well with the survival data in that groups of animals vaccinated with recombinant viruses that induced high levels of protection (vP829, vP555, and

TABLE 1

NEUT <sup>a</sup> AND HAI ANTIBODY TITERS IN PRECHALLENGE SERA				
Immunizing virus <sup>a</sup>	One inoculation		Two inoculations	
	NEUT titer <sup>b</sup>	HAI titer <sup>c</sup>	NEUT titer <sup>b</sup>	HAI titer <sup>c</sup>
vP410 Group 1	<1:10	<1:10		
vP410 Group 2	<1:10	<1:10	<1:10	<1:10
vP555 Group 1	1:20	1:20		
vP555 Group 2	1:20	1:20	1:320	1:80
vP825 Group 1	1:10	1:10		
vP825 Group 2	<1:10	1:10	1:320	1:40
vP829 Group 1	1:80	1:40		
vP829 Group 2	1:160	1:40	1:2560	1:160
vP857 Group 1	<1:10	<1:10		
vP857 Group 2	<1:10	<1:10	<1:10	<1:10
vP864 Group 1	<1:10	<1:10		
vP864 Group 2	<1:10	<1:10	<1:10	<1:10

<sup>a</sup> Vaccinia recombinant used for immunization; Group 1 indicates mice challenged 3 weeks following a single vaccinia inoculation, and Group 2 indicates mice challenged following two inoculations (see Materials and Methods). JEV sequences incorporated in the recombinant viruses are shown in Fig. 2; vP410 is the parental virus.

<sup>b</sup> Serum dilution yielding 90% reduction in plaque number.

<sup>c</sup> Serum dilution.

TABLE 2  
SURVIVAL OF VACCINIA-IMMUNIZED MICE FOLLOWING  
A LETHAL JEV CHALLENGE

Immunizing virus <sup>a</sup>	Survival after one inoculation <sup>b</sup>	Survival after two inoculations <sup>c</sup>
vP410	0/10	0/10
vP555	7/10	10/10
vP825	8/10	9/10
vP829	10/10	9/10
vP857	0/10	5/10
vP864	1/10	6/10

<sup>a</sup> Vaccinia recombinant used for immunization. JEV sequences incorporated in the recombinant viruses are shown in Fig. 2; vP410 is the parental virus.

<sup>b</sup> Live animals/total for each group; challenged with  $4.9 \times 10^5$  LD<sub>50</sub> of JEV 3 weeks following a single vaccinia inoculation.

<sup>c</sup> Live animals/total for each group; challenged with  $1.3 \times 10^3$  LD<sub>50</sub> of JEV 6 weeks following the first inoculation and 3 weeks following the second inoculation with the same vaccinia recombinant.

vP825) showed low postchallenge responses to NS3, whereas the sera from survivors in groups vaccinated with recombinants that expressed NS1 alone (vP857 and vP864) showed much higher postchallenge responses to NS3 (Fig. 6). In addition to responding to the NS3 protein, mice vaccinated with recombinants expressing only NS1 (vP857 and vP864) showed strong postchallenge responses to E, and mice vaccinated with vP829 which expresses prM and E showed postchallenge responses to NS1. Furthermore, overexposure of the gel shown in Fig. 6 showed specific reaction to NS3, even in animals inoculated two times with vP829.

## DISCUSSION

We constructed four new JEV-vaccinia recombinant viruses to analyze the contribution of individual JEV antigens to protective immunity and to further characterize the biosynthesis of the JEV proteins. Evaluation of these recombinant viruses in a mouse challenge system showed that viruses encoding NS1 alone provided some protection from lethal infection, but much better protection was achieved with viruses that induced neutralizing antibodies. Especially high neutralizing antibody titers were observed in mice inoculated with the recombinant vP829, which produced SHA-like extracellular particles containing E. This recombinant virus serves as our best vaccine candidate to date and should be a useful tool for studying assembly of flavivirus structural proteins.

Several reports from other laboratories have documented the ability of flavivirus-vaccinia recombinants

to protect mice from lethal flavivirus challenge. Yasuda *et al.* (1990) have shown that recombinant viruses expressing the prM and E proteins of JEV induce neutralizing antibodies and protect mice from approximately 10 LD<sub>50</sub> of challenge virus. Lai and co-workers have shown that a recombinant containing the entire 5' end of the dengue type 4 viral ORF (extending from C to NS2A) produce intracellular forms of prM, E, and NS1 but fail to induce the synthesis of extracellular forms of any of the structural proteins (Zhao *et al.*, 1987; Bray *et al.*, 1989). Additional dengue 4 recombinants that contained several forms of the E gene have also been examined, and although several of these recombinant viruses were protective, they did not produce extracellular forms of E (Bray *et al.*, 1989). Interestingly, a dengue-vaccinia recombinant expressing a C-terminally truncated E protein gene induced the synthesis of an extracellular form of E and provided an increased level of resistance to dengue virus encephalitis in inoculated mice (Men *et al.*, 1991).

Some vaccinia recombinants have yielded less encouraging results. Dengue type 2 structural proteins have been expressed by recombinant vaccinia viruses (Deubel *et al.*, 1988), and although these viruses induced the synthesis of the structural glycoprotein within infected cells, they neither elicited detectable anti-dengue immune responses nor protected monkeys from dengue infection. Recombinants constructed to express structural proteins of dengue type 2 or yellow fever virus elicited low and variable levels of neutralizing antibodies in inoculated mice (Hahn *et al.*,

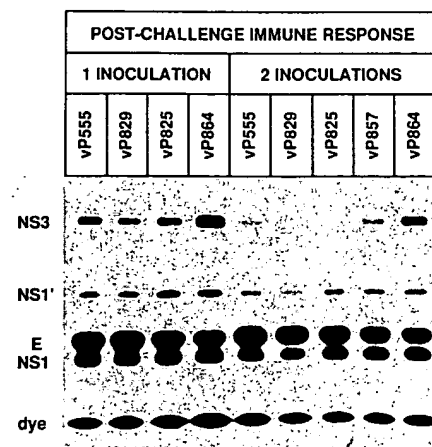


FIG. 6. Analysis of the JEV-specific reactivity of sera obtained from mice that survived JEV challenge. Sera collected from all animals in the indicated groups that survived challenge (see Tables 1 and 2) were pooled and aliquots were tested for their ability to immunoprecipitate radiolabeled proteins harvested from JEV-infected cells. The positions of JEV proteins are shown at the left side of the autoradiogram.

1990). Mice immunized with a recombinant expressing NS1 of yellow fever virus were partially protected against lethal challenge with yellow fever virus (Putnak and Schlesinger, 1990).

Our previous report on vaccinia recombinants that produced extracellular SHA-like particles suggested that prM was required for production of these particles since they were only observed in the culture fluids of cells infected with vaccinias that expressed prM in addition to E and NS1 (Mason *et al.*, 1991). In this study we have shown that a recombinant containing the C coding region in addition to prM and E (vP825) failed to produce any extracellular forms of the structural proteins. Thus, we speculate that the factors needed for the production of extracellular SHA-like particles are (1) the possession of coding regions for prM and E and (2) the absence of the C coding region. Although we have not formally shown that vP825-infected cells contain any form(s) of C, we feel that the expression of C in the absence of other viral components (including viral proteases and RNA) could interfere with the assembly of the viral membrane proteins into a particulate form.

The recombinant viruses, vP555, vP857, and vP864, induced the synthesis of mature extracellular forms of NS1. However, cells infected with the recombinant vP825 that has the JEV ORF insert from C to NS2A released barely detectable amounts of NS1 into the culture fluid. This reduction in release of NS1 in cells that accumulate E and prM suggests that NS1 could be bound to the structural proteins during some stage of viral envelope assembly. Moreover, the release of higher hemagglutination activity from vP829-infected than vP555-infected cells may be related to the retardation of the movement of subviral particles (SHA) through the secretory pathway in the presence of NS1.

Our previous studies showed that viruses that produced extracellular forms of the structural proteins of JEV protected mice from lethal JEV infection (Mason *et al.*, 1991). The present study demonstrates that recombinants which synthesize the most extracellular particles *in vitro* elicit the highest NEUT antibody titers in mice. These results suggest that the synthesis of extracellular SHA-like particles is important for eliciting high levels of protective immunity in mice. However production of extracellular particles *in vitro* is not an absolute requisite for protection since vP825, which does not induce the production of an extracellular HA in infected cells, induces NEUT antibodies and protects mice. The ability of vP825 to protect animals from lethal infection, whereas vP583 or vP658 (expressing E and NS1; Mason *et al.*, 1991) did not protect, suggests that infected cell-associated E is an effective immunogen when produced in the presence of prM. The ability

of this cell-associated form of E to immunize animals could be due to stabilization of the E antigen by prM following synthesis or during lysis of the vaccinia recombinant-infected cells *in vivo*.

One of the goals of this study was to evaluate the ability of recombinants expressing NS1 alone to protect mice from JEV encephalitis. These experiments show that recombinants, vP857, and vP864, which induce the synthesis of NS1 protein, induced antibodies to NS1 in inoculated mice. However, these recombinants were much less effective than structural protein expressing recombinants in protecting mice from JEV encephalitis. The radioimmunoprecipitation data shown in Fig. 6 demonstrate higher antibody levels to NS3 in postchallenge sera obtained from mice immunized with recombinants expressing NS1 than those coexpressing prM and E. This finding supports the idea that NEUT antibody prevents virus replication more effectively than antibody to NS1. Interestingly, antibodies to NS3 were detectable in pooled sera from vP555-, vP825-, or vP829-immunized mice which had neutralizing antibodies prior to challenge. These results demonstrate that some challenge virus replication occurred in these animals despite the fact that the animals were protected from JEV-induced disease.

## ACKNOWLEDGMENTS

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## REFERENCES

- BRAY, M., ZHAO, B., MARKOFF, L., ECKELS, K. H., CHANOCK, R. M., and LAI, C.-J. (1989). Mice immunized with recombinant vaccinia virus expressing dengue 4 virus structural proteins with or without non-structural protein NS1 are protected against fatal dengue virus encephalitis. *J. Virol.* 63, 2853-2856.
- CANE, P. A., and GOULD, E. A. (1988). Reduction of yellow fever virus mouse neurovirulence by immunization with a bacterially synthesized non-structural protein (NS1) fragment. *J. Gen. Virol.* 69, 1241-1246.
- CHAMBERS, T. J., HAHN, C. S., GALLER, R., and RICE, C. M. (1990). Flavivirus genome organization, expression, and replication. *Annu. Rev. Microbiol.* 44, 649-688.
- D'ALESSIO, J. M., and GERARD, G. F. (1988). Second-strand cDNA synthesis with *E. coli* DNA polymerase I and RNase H: The fate of

- information at the mRNA 5' terminus and the effect of *E. coli* DNA ligase. *Nucleic Acids Res.* 16, 1999–2014.
- DEUBEL, V., BORDIER, M., MEGRET, F., GENTRY, M. K., SCHLESINGER, J. J., and GIRARD, M. (1991). Processing, secretion, and immunoreactivity of carboxy terminally truncated dengue-2 virus envelope proteins expressed in insect cells by recombinant baculoviruses. *Virology* 180, 442–447.
- DEUBEL, V., KINNEY, R. M., ESPOSITO, J. J., CROPP, C. B., VORNDAM, A. V., MONATH, T. P., and TRENT, D. (1988). Dengue 2 virus envelope protein expressed by a recombinant vaccinia virus fails to protect monkeys against dengue. *J. Gen. Virol.* 69, 1921–1929.
- FALGOUT, B., BRAY, M., SCHLESINGER, J. J., and LAI, C.-J. (1990). Immunization of mice with recombinant vaccinia virus expressing authentic dengue virus nonstructural protein NS1 protects against lethal dengue virus encephalitis. *J. Virol.* 64, 4356–4363.
- FALGOUT, B., CHANOCK, R., and LAI, C.-J. (1989). Proper processing of dengue virus nonstructural glycoprotein NS1 requires the N-terminal hydrophobic signal sequence and the downstream nonstructural protein NS2a. *J. Virol.* 63, 1852–1860.
- GOULD, E. A., BUCKLEY, A., BARRETT, A. D. T., and CAMMACK, N. (1986). Neutralizing (54K) and non-neutralizing (54K and 48K) monoclonal antibodies against structural and non-structural yellow fever virus proteins confer immunity in mice. *J. Gen. Virol.* 67, 591–595.
- GUO, P., GOEBEL, S., DAVIS, S., PERKUS, M. E., LANGUET, B., DESMETTRE, P., ALLEN, G., and PAOLETTI, E. (1989). Expression in recombinant vaccinia virus of the equine herpesvirus 1 gene encoding glycoprotein gp13 and protection of immunized animals. *J. Virol.* 63, 4189–4198.
- HAHN, Y. S., LENCHES, E. M., GALLER, R., RICE, C. M., DALRYMPLE, J., and STRAUSS, J. H. (1990). Expression of the structural proteins of dengue 2 virus and yellow fever virus by recombinant vaccinia viruses. *Arch. Virol.* 115, 251–265.
- HAISHI, S., IMAI, H., HIRAI, K., IGARASHI, A., and KATO, S. (1989). Expression of envelope glycoprotein (E) of Japanese encephalitis virus by recombinant vaccinia virus. *Acta Virol.* 33, 497–503.
- HALSTEAD, S. B. (1988). Pathogenesis of dengue: challenges to molecular biology. *Science* 239, 476–481.
- HEINZ, F. X., BERGER, R., TUMA, W., and KUNZ, C. (1983). A topological and functional model of epitopes on the structural glycoprotein of tick-borne encephalitis virus defined by monoclonal antibodies. *Virology* 126, 525–537.
- HENCHAL, E. A., HENCHAL, L. S., and SCHLESINGER, J. J. (1988). Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. *J. Gen. Virol.* 69, 2101–2107.
- HUANG, C. H. (1982). Studies of Japanese encephalitis in China. *Adv. Virus Res.* 27, 71–101.
- IGARASHI, A. (1978). Isolation of a Singh's *Aedes albopictus* cell clone sensitive to dengue and chikungunya viruses. *J. Gen. Virol.* 40, 531–544.
- KAUFMAN, B. M., SUMMERS, P. L., DUBOIS, D. R., COHEN, W. H., GENTRY, M. K., TIMCHAK, R. L., BURKE, D. S., and ECKELS, K. H. (1989). Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. *Am. J. Trop. Med. Hyg.* 41, 576–580.
- KAUFMAN, B. M., SUMMERS, P. L., DUBOIS, D. R., and ECKELS, K. H. (1987). Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. *Am. J. Trop. Med. Hyg.* 36, 427–434.
- KIMURA-KURODA, J., and YASUI, K. (1988). Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies. *J. Immunol.* 141, 3606–3610.
- MANDECKI, W. (1986). Oligonucleotide-directed double-strand break repair in plasmids of *Escherichia coli*: A method for site-specific mutagenesis. *Proc. Natl. Acad. Sci. USA* 83, 7177–7181.
- MANIATIS, T., FRITSCH, E. F., and SAMBROOK, J. (1982). "Molecular Cloning: A Laboratory Manual: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- MASON, P. W. (1989). Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology* 169, 354–364.
- MASON, P. W., DALRYMPLE, J. M., GENTRY, M. K., MCCOWN, J. M., HOKE, C. H., BURKE, D. S., FOURNIER, M. J., and MASON, T. L. (1989). Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. *J. Gen. Virol.* 70, 2037–2049.
- MASON, P. W., MCADA, P. C., DALRYMPLE, J. M., FOURNIER, M. J., and MASON, T. L. (1987). Expression of Japanese encephalitis virus antigens in *Escherichia coli*. *Virology* 158, 361–372.
- MASON, P. W., PINCUS, S., FOURNIER, M. J., MASON, T. L., SHOPE, R. E., and PAOLETTI, E. (1991). Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. *Virology* 180, 294–305.
- MATHEWS, J. H., and ROEHRIG, J. T. (1984). Elucidation of the topography and determination of the protective epitopes on the E glycoprotein of Saint Louis encephalitis virus by passive transfer with monoclonal antibodies. *J. Immunol.* 132, 1533–1537.
- MATSUURA, Y., MIYAMOTO, M., SATO, T., MORITA, C., and YASUI, K. (1989). Characterization of Japanese encephalitis virus envelope protein expressed by recombinant baculoviruses. *Virology* 173, 674–682.
- MCADA, P. C., MASON, P. W., SCHMALJOHN, C. S., DALRYMPLE, J. M., MASON, T. L., and FOURNIER, M. J. (1987). Partial nucleotide sequence of the Japanese encephalitis virus genome. *Virology* 158, 348–360.
- MCCOWN, J., COCHRAN, M., PUTNAK, R., FEIGHNY, R., BURROUS, J., HENCHAL, E., and HOKE, C. (1990). Protection of mice against lethal Japanese encephalitis with a recombinant baculovirus vaccine. *Am. J. Trop. Med. Hyg.* 42, 491–499.
- MEN, R., BRAY, M., and LAI, C.-J. (1991). Carboxy-terminally truncated dengue virus envelope glycoproteins expressed on the cell surface and secreted extracellularly exhibit increased immunogenicity in mice. *J. Virol.* 65, 1400–1407.
- MONATH, T. P. (1986). Pathobiology of the Flaviviruses. In "The Togaviridae and Flaviviridae" (S. Schlesinger and M. J. Schlesinger, Eds.), Plenum Press, New York/London. Pp. 375–440.
- OKAYAMA, H., and BERG, P. (1982). High-efficiency cloning of full-length cDNA. *Mol. Cell. Biol.* 2, 161–170.
- PANICALI, D., and PAOLETTI, E. (1982). Construction of poxviruses as cloning vectors: Insertion of the thymidine kinase gene from herpes simplex virus into the DNA of an infectious vaccinia virus. *Proc. Natl. Acad. Sci. USA* 79, 4927–4931.
- PUTNAK, J. R., and SCHLESINGER, J. J. (1990). Protection of mice against yellow fever virus encephalitis by immunization with a vaccinia virus recombinant encoding the yellow fever virus nonstructural proteins, NS1, NS2a and NS2b. *J. Gen. Virol.* 71, 1697–1702.
- REPIK, P. M., DALRYMPLE, J. M., BRANDT, W. E., MCCOWN, J. M., and RUSSELL, P. K. (1983). RNA fingerprinting as a method for distinguishing dengue 1 virus strains. *Am. J. Trop. Med. Hyg.* 32, 577–589.
- SANGER, F., NICKLEN, S., and COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74, 5463–5467.
- SCHLESINGER, J. J., BRANDRISS, M. W., CROPP, C. B., and MONATH, T. P. (1986). Protection of mice against Japanese encephalitis virus by immunization with a vaccinia virus recombinant encoding the yellow fever virus nonstructural proteins, NS1, NS2a and NS2b. *J. Gen. Virol.* 67, 1697–1702.

- T. P. (1986). Protection against yellow fever in monkeys by immunization with yellow fever virus nonstructural protein NS1. *J. Virol.* 60, 1153-1155.
- SCHLESINGER, J. J., BRANDRISS, M. W., and WALSH, E. E. (1985). Protection against 17D yellow fever encephalitis in mice by passive transfer of monoclonal antibodies to the nonstructural glycoprotein gp48 and by active immunization with gp48. *J. Immunol.* 135, 2805-2809.
- SCHLESINGER, J. J., BRANDRISS, M. W., and WALSH, E. E. (1987). Protection of mice against dengue 2 virus encephalitis by immunization with the dengue 2 virus non-structural glycoprotein NS1. *J. Gen. Virol.* 68, 853-857.
- SHOPE, R. E. (1980). Medical Significance of Togaviruses: An Overview of Diseases Caused by Togaviruses in Man and in Domestic and Wild Vertebrate Animals. In "The Togaviruses" (R. W. Schlesinger, Ed.), Academic Press, NY. Pp. 47-82.
- YASUDA, A., KIMURA-KURODA, J., OGIMOTO, M., MIYAMOTO, M., SATA, T., SATO, T., TAKAMURA, C., KURATA, T., KOJIMA, A., and YASUI, K. (1990). Induction of protective immunity in animals vaccinated with recombinant vaccinia viruses that express preM and E glycoproteins of Japanese encephalitis virus. *J. Virol.* 64, 2788-2795.
- YUEN, L., and MOSS, B. (1987). Oligonucleotide sequence signaling transcriptional termination of vaccinia virus early genes. *Proc. Natl. Acad. Sci. USA* 84, 6417-6421.
- ZHANG, Y.-M., HAYES, E. P., MCCARTHY, T. C., DUBOIS, D. R., SUMMERS, P. L., ECKELS, K. H., CHANOCK, R. M., and LAI, C.-J. (1988). Immunization of mice with dengue structural proteins and non-structural protein NS1 expressed by baculovirus recombinant induces resistance to Dengue virus encephalitis. *J. Virol.* 62, 3027-3031.
- ZHAO, B., PRINCE, G., HORSWOOD, R., ECKELS, K., SUMMERS, P., CHANOCK, R., and LAI, C.-J. (1987). Expression of dengue virus structural proteins and non-structural protein NS1 by a recombinant vaccinia virus. *J. Virol.* 61, 4019-4022.



## Proper Maturation of the Japanese Encephalitis Virus Envelope Glycoprotein Requires Cosynthesis with the Premembrane Protein

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The role of the Japanese encephalitis virus (JEV) premembrane (prM) protein in maturation of the envelope (E) glycoprotein was evaluated by using recombinant vaccinia viruses encoding E in the presence (vP829) or absence (vP658) of prM. Immunofluorescence analyses showed that E appeared to be localized in the endoplasmic reticulum of cells infected with JEV, vP829, or vP658. However, reactivity with monoclonal antibodies and behavior in Triton X-114 indicated that E produced in the absence of prM behaved abnormally. Furthermore, E produced in the presence of prM by recombinant vaccinia viruses could be incorporated into flavivirus pseudotypes, whereas E synthesized in the absence of prM could not. These results demonstrate that cosynthesis of prM is required for proper folding, membrane association, and assembly of the flavivirus E protein.

The flavivirus virion consists of a nucleocapsid surrounded by a lipid bilayer containing an envelope (E) glycoprotein and a nonglycosylated membrane (M) protein. The M protein is found in infected cells as a glycosylated precursor, prM, which is cleaved to form M in a late-stage cleavage event, presumably by a cellular protease located in the secretory pathway (reviewed in reference 3). The prM and E proteins are synthesized as a part of a polyprotein processed during translation through the endoplasmic reticulum (ER) membrane. These proteins appear inside the lumen of the ER with their N termini cleaved by signalase, and their C-terminal hydrophobic amino acid domains supply stop transfer sequences and membrane anchors. The E protein plays an important role in receptor binding and membrane fusion and contains most of the sites that react with neutralizing antibodies as well as many protective epitopes (reviewed in reference 7). The roles for prM in virion function and viral biology remain unclear, although prM is probably involved in virion maturation, since cleavage of prM to M is associated with changes in infectivity (14) and fusion activity (6) and since prM and E are found in cell-associated heterodimers (14).

We have developed recombinant vaccinia viruses encoding various combinations of Japanese encephalitis virus (JEV) structural and nonstructural (NS) proteins in order to define the roles of these proteins in inducing protective immunity (8, 13). Two of these viruses, vP555 and vP829, encode prM and E and induced the proper synthesis of both intracellular and extracellular forms of the prM/M and E proteins in infected cells (8, 13), in the presence (vP555) or absence (vP829) of NS1. Furthermore, we demonstrated that cells infected with vP829 released subviral particles of 20-nm diameter that were characterized as flavivirus RNA-free membrane vesicles with prM/M and E embedded in a lipid bilayer (9). Another recombinant, vP658, encodes E and

NS1, and E produced by cells infected with this virus is not released into the extracellular fluid (13).

To determine whether E produced by vP658 was not released from cells because of its presence in a different subcellular compartment, we used indirect immunofluorescence microscopy. The results of these studies (Fig. 1) showed that E was distributed in a fine network in the cytoplasm of cells infected with either vP658 or vP829, suggesting that E produced in cells infected with either recombinant is present in the ER, consistent with previous N-linked glycosylation studies (8, 13).

To further probe the differences in the E protein produced by cells infected with these viruses, we compared the reactions of E prepared from cells infected with the Nakayama strain of JEV, vP829, or vP658 with a panel of monoclonal antibodies (MAbs). For these analyses, radioactive antigens prepared from cells infected with JEV, vP829, or vP658 were immunoprecipitated with MAbs and subjected to sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis (SDS-PAGE), and the amount of E precipitated was determined by using a Molecular Dynamics PhosphorImager. Table 1 shows the results for three selected MAbs. Two of these, J3-11G5 and J3-14E6 (12), are shown since they exhibited the highest and lowest reactions with the authentic (JEV) E protein, and the third MAb, D1-4G2 (5), was selected since it reacts with a discontinuous epitope and its binding is dependent on correct disulfide bond formation within E (12). These results indicate that the vP658-encoded E was present in cell lysates in much lower amounts, probably as a result of degradation since identical vectors and promoters were used for construction of vP829 and vP658 (8, 13). Furthermore, the vP658 E is in an antigenically different conformation from E present in cells infected with JEV or vP829, since it reacted very poorly with D1-4G2.

The hydrophobic properties of E produced by JEV, vP829, and vP658-infected cells were compared by using Triton X-114 phase separation experiments. As shown in Fig. 2, most of the radioactive E obtained from JEV- and

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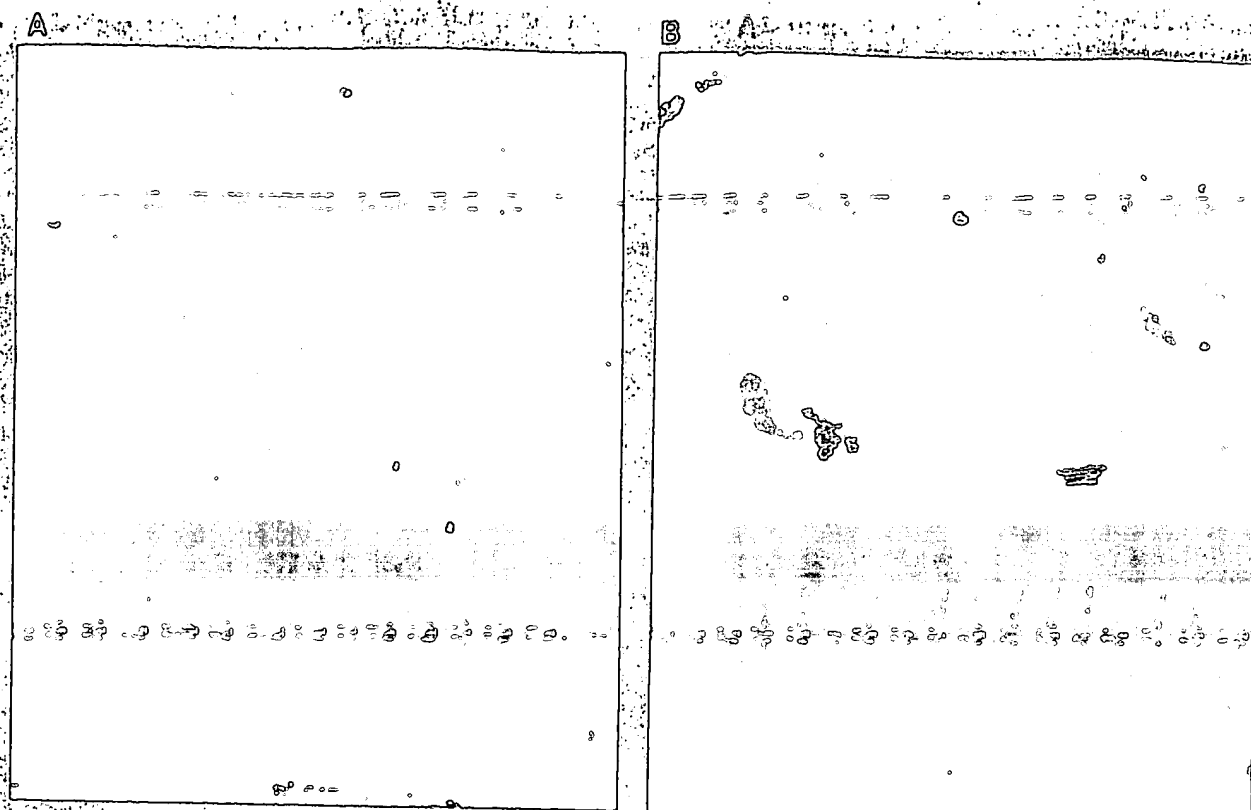


FIG. 1. Immunofluorescence micrographs of HeLa cells infected with vP829 (A) and vP658 (B). Cell monolayers were infected with recombinant vaccinia viruses (multiplicity of infection of 2); 24 h later, the monolayers were fixed with cold ethanol and stained with a MAb against E (J3-11B9) and a goat anti-mouse fluorescein isothiocyanate-conjugated antibody.

vP829-infected cells partitioned into the detergent phase, whereas more than half of the E protein molecules synthesized in vP658-infected cells were found in the aqueous phase. Furthermore, essentially 100% of E found in culture fluids of JEV- and vP829-infected cells remained in the detergent phase during extraction, and no E was released from vP658-infected cells, confirming earlier studies (13). These results suggest that E synthesized in vP658-infected cells was improperly folded, preventing it from displaying the amphipathic properties of E produced by JEV- and vP829-infected cells. The hydrophilic forms of E found in vP658-infected cells are probably similar to misfolded disul-

fide-linked aggregates of newly synthesized viral glycoproteins recently identified by Marquardt and Helenius (10). The E protein produced by vP658 is present in similar types of aggregates, since most of the E protein found in vP658-infected cells does not enter the separatory gel in SDS-PAGE in the absence of reduction (data not shown).

Production of pseudotype viruses by using vector-encoded proteins in cells coinfecting with related viruses has recently been used to assay oligomerization of the rhabdovirus E glycoprotein (15). We have used a similar approach to determine whether E produced by our recombinant vaccinia viruses could be incorporated into flavivirus pseudotypes.

TABLE 1. Reaction of E produced by JEV, vP829, and vP658-infected HeLa cells with MAbs<sup>a</sup>

Virus	Relative amt <sup>b</sup> after immunoprecipitation with MAb:		
	J3-11G5	J3-14E6	D1-4G2
JEV	57	35	39
vP829	95	60	55
vP658	8.8	5.6	1.1

<sup>a</sup> HeLa cell monolayers were infected with recombinant vaccinia viruses (multiplicity of infection of 2) or JEV (multiplicity of infection of 5) and then were pulse-labeled for 2 h with [<sup>35</sup>S]methionine and chased for 6 h in the presence of excess unlabeled methionine (13). Radiolabeled cell lysates and culture fluids were immunoprecipitated with MAbs against E, J3-11G5, J3-14E6 (12), and D1-4G2 (5), and then resolved by SDS-PAGE.

<sup>b</sup> Relative amounts of immunoprecipitated E protein present in SDS-polyacrylamide gels determined with a PhosphorImager.

CELL LYSATE				CULTURE FLUID			
JEV	vP829	vP658		JEV	vP829	vP658	
A	D	A	D	A	D	A	D

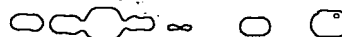


FIG. 2. Radioimmunoprecipitates of Triton X-114-extracted lysates of HeLa cells infected with JEV, vP829, or vP658. Cell monolayers and culture fluids obtained from [<sup>35</sup>S]Met-labeled, infected cells were subjected to phase separation by using 2% Triton X-114 prepared in 50 mM Tris-HCl (pH 7.5) containing protease inhibitors as previously described (2, 11); E was immunoprecipitated from equal portions of the aqueous (A) and washed detergent (D) phases by using MAb J3-11B9, subjected to SDS-PAGE, and autoradiographed.

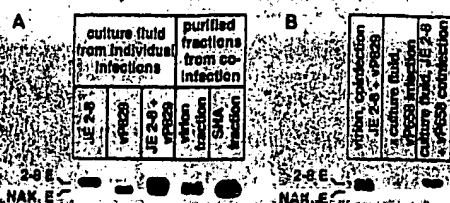


FIG. 3. Results of assays using a pseudotype virus containing E proteins of two different molecular weights that was produced by HeLa cells dually infected with the 2-8 strain of JEV and a recombinant vaccinia virus, vP829 (both at a multiplicity of infection of 5). (A) Immunoprecipitates from culture fluids obtained from cells infected singly with the 2-8 strain of JEV or vP829 electrophoresed either separately or together as a mixture, as indicated. The two lanes on the right show the immunoprecipitates prepared from the sucrose density gradient-purified virion and slowly sedimenting hemagglutinin (SHA) fractions obtained from culture fluids of cells coinfecting with 2-8 and vP829. (B) Immunoprecipitates prepared from the virion fraction obtained from cells coinfecting with 2-8 and vP829, from the culture fluid of cells infected with vP658 alone, or from the culture fluid from cells coinfecting with 2-8 and vP658, as indicated. In all cases, immunoprecipitates were digested with 0.4 U of glycoproteinase F prior to SDS-PAGE (11).

Biochemical evidence for JEV pseudotype formation was obtained by evaluation of E in virion fractions obtained from cells coinfecting with the recombinant vaccinia viruses and the 2-8 strain of JEV (4; obtained from the Yale Arbovirus Research Unit), which produces an E protein with an apparent higher molecular weight. Figure 3A shows that sucrose density gradient-purified virions produced by dual infection with the 2-8 strain and vP829 contained E proteins corresponding in size to E produced by the 2-8 strain and by vP829. This result indicates that E encoded by vP829 had been incorporated into the 2-8 virion, demonstrating pseudotype formation in cells coinfecting with vP829. In contrast, culture fluids of cells dually infected with the 2-8 strain and vP658 showed only a single band corresponding to E encoded by the 2-8 strain (Fig. 3B), indicating that virions produced by coinfection with vP658 did not possess the E protein encoded by vP658; even overexposure of the gel failed to show any evidence for the Nakayama-sized E protein in this sample. Furthermore, the absence of any vaccinia virus-derived E in the culture fluid indicates that the prM protein synthesized by 2-8 could not rescue the vaccinia virus-encoded E protein.

The production of flavivirus pseudotypes by coinfection with recombinant vaccinia viruses was further examined by using a related flavivirus, Murray Valley encephalitis virus (MVEV; obtained from the Yale Arbovirus Research Unit), and neutralization tests. Surprisingly, viruses harvested from cells dually infected with MVEV and vP829 could be neutralized with MAb J3-14H5 at a dose-response level identical to that for JEV. This efficient neutralization probably reflects an efficient incorporation of the overexpressed JEV E protein into the resulting viruses and may also reflect the ability of this MAb to neutralize virus by binding to only a small number of sites on the virion, consistent with its high neutralization titer (12). No detectable neutralization with this JEV-specific MAb was noted in the virus harvested from cells dually infected with MVEV and vP658 (Fig. 4). These results show that JEV E cosynthesized with prM was capable of being incorporated into the MVEV virion, whereas JEV E produced in the absence of prM was not.

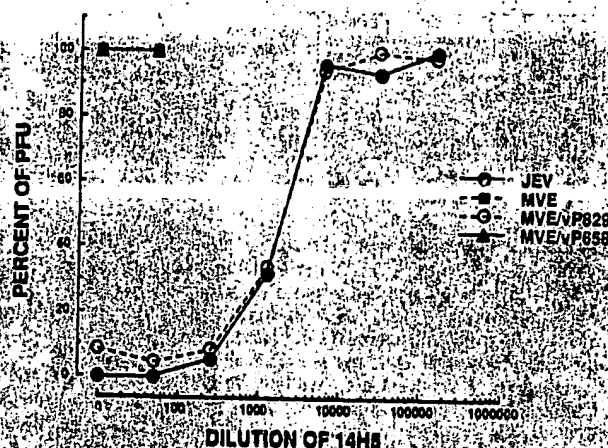


FIG. 4. Neutralization of virus produced by dual infection of HeLa cells with MVEV and recombinant vaccinia viruses. Cells were infected with MVEV (multiplicity of infection of 2), incubated for 24 h, and superinfected with vP829 or vP658 (multiplicity of infection of 5). The medium was renewed 16 h later, and the sucrose density gradient-purified virion fractions prepared from culture fluid harvested 8 h later were tested for neutralization by using the JEV-specific MAb J3-14H5 (12), in the presence of mouse anti-wild-type vaccinia virus (added to inhibit vaccinia virus plaque formation). Data are expressed as percentage of PFU obtained in the absence of the MAb.

The finding that dual infection with vP658 failed to produce pseudotypes is consistent with the conclusion presented above that E produced in the absence of prM accumulates in an aggregated, antigenically altered form in the ER. Thus, proper folding, maturation, and assembly of E require co-synthesis with prM.

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#### REFERENCES

1. Bangs, J. D., N. W. Andrews, G. W. Hart, and P. T. Englund. 1986. Posttranslational modification and intracellular transport of a trypanosome variant surface glycoprotein. *J. Cell. Biol.* 103:255-263.
2. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604-1607.
3. Chambers, T. J., C. S. Hahn, R. Galler, and C. M. Rice. 1990. Flavivirus genome organization, expression, and replication. *Annu. Rev. Microbiol.* 44:649-688.
4. Chen, B. Q., and B. J. Beaty. 1982. Japanese encephalitis vaccine (2-8 strain) and parent (SA 14 strain) viruses in *Culex tritaeniorhynchus* mosquitoes. *Am. J. Trop. Med. Hyg.* 31:403-407.
5. Gentry, M. K., E. A. Henchal, J. M. McCown, W. E. Brandt, and J. M. Dalrymple. 1982. Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies. *Am. J. Trop. Med. Hyg.* 31:548-555.
6. Guirakhoo, F., F. X. Heinz, C. W. Mandl, H. Holzmann, and C. Kunz. 1991. Fusion activity of flaviviruses: comparison of mature and immature (prM-containing) tick-borne encephalitis virions. *J. Gen. Virol.* 72:1323-1329.
7. Heinz, F. X. 1986. Epitope mapping of flavivirus glycoproteins.

Adv. Virus Res. 31:103-168.

8. Konishi, E., S. Pincus, B. A. L. Fonseca, R. E. Shope, E. Paoletti, and P. W. Mason. 1991. Comparison of protective immunity elicited by recombinant vaccinia viruses that synthesize E or NS1 of Japanese encephalitis virus. *Virology* 185:401-410.
9. Konishi, E., S. Pincus, E. Paoletti, R. E. Shope, T. Burrage, and P. W. Mason. 1992. Mice immunized with a sub-viral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. *Virology* 188:714-720.
10. Marquardt, T., and A. Helenius. 1992. Misfolding and aggregation of newly synthesized proteins in the endoplasmic reticulum. *J. Cell Biol.* 117:505-513.
11. Mason, P. W. 1989. Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. *Virology* 169:354-364.
12. Mason, P. W., J. M. Dalrymple, M. K. Gentry, J. M. McCown, C. H. Hoke, D. S. Burke, M. J. Fournier, and T. L. Mason. 1989. Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. *J. Gen. Virol.* 70:2037-2049.
13. Mason, P. W., S. Pincus, M. J. Fournier, T. L. Mason, R. E. Shope, and E. Paoletti. 1991. Japanese encephalitis virus-vaccinia recombinants produce particulate forms of the structural membrane proteins and induce high levels of protection against lethal JEV infection. *Virology* 180:294-305.
14. Wengler, G., and G. Wengler. 1989. Cell-associated West Nile flavivirus is covered with E + pre-M protein heterodimers which are destroyed and reorganized by proteolytic cleavage during virus release. *J. Virol.* 63:2521-2526.
15. Whitt, M. A., L. Buonocore, C. Prehaud, and J. K. Rose. 1991. Membrane fusion activity, oligomerization, and assembly of the rabies virus glycoprotein. *Virology* 185:681-689.

# Point Mutations Define a Sequence Flanking the AUG Initiator Codon That Modulates Translation by Eukaryotic Ribosomes

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## Summary

By analyzing the effects of single base substitutions around the ATG initiator codon in a cloned preproinsulin gene, I have identified ACCATGG as the optimal sequence for initiation by eukaryotic ribosomes. Mutations within that sequence modulate the yield of proinsulin over a 20-fold range. A purine in position -3 (i.e., 3 nucleotides upstream from the ATG codon) has a dominant effect; when a pyrimidine replaces the purine in position -3, translation becomes more sensitive to changes in positions -1, -2, and +4. Single base substitutions around an upstream, out-of-frame ATG codon affect the efficiency with which it acts as a barrier to initiating at the downstream start site for preproinsulin. The optimal sequence for initiation defined by mutagenesis is identical to the consensus sequence that emerged previously from surveys of translational start sites in eukaryotic mRNAs. The mechanism by which nucleotides flanking the ATG codon might exert their effect is discussed.

## Introduction

A considerable body of evidence supports the idea that 40S ribosomal subunits bind at the capped 5' end and scan the mRNA sequence until an AUG codon is reached (Kozak, 1980a, 1983a). The fact that 40S subunits can migrate on mRNA, prior to the assembly of a complete 80S ribosome, has been demonstrated *in vitro* (Kozak and Shatkin, 1978; Kozak, 1980b). The stimulatory effect of the m<sup>7</sup>G cap (Shatkin, 1976) and the inability of ribosomes to bind to circular mRNAs (Kozak, 1979; Konarska et al., 1981) point to an end-dependent mechanism. The fact that eukaryotic ribosomes usually translate only the 5'-proximal cistron in polycistronic viral mRNAs (Smith, 1977) is also rationalized by the scanning model. The importance of position in defining the functional initiation site was shown by manipulating a cloned preproinsulin gene to produce an mRNA in which the "ribosome binding site" (i.e. the ATG initiator codon and flanking sequence) was tandemly reiterated: ribosomes initiated exclusively at the 5'-proximal copy in the tandem array (Kozak, 1983b).

Inspection of sequences near the 5' ends of eukaryotic mRNAs provides evidence that might be interpreted for or against the scanning hypothesis: In ~80% of the mRNAs examined, there are no extraneous AUG triplets upstream of the functional initiator codon—a provocative finding that is rationalized by the scanning model. However, 5% to 10% of eukaryotic mRNAs have AUG triplet(s) upstream of the known start site for protein synthesis (Kozak,

1983a). In such mRNAs, the upstream AUG triplets occur in a context different from the conserved pattern of nucleotides around functional initiator codons (Kozak, 1981, 1983a, 1984a). This difference inspired a modified version of the scanning model in which both the position of an AUG codon and its context play a role (Kozak, 1981). Our current working hypothesis is that a 40S ribosomal subunit (with associated factors, of course) binds at the 5' end of mRNA and advances linearly until it reaches the first AUG triplet: if the first AUG codon occurs in an optimal context, all 40S subunits stop and that AUG serves as the unique site of initiation. But if the sequence around the first AUG triplet is suboptimal, some 40S subunits bypass that site and initiate farther downstream. The optimal context for initiation, derived from the aforementioned survey, was CCACCAUGG (Kozak, 1981, 1984a). Within that sequence, the purine in position -3 (3 nucleotides upstream of the AUG codon) is most highly conserved: ~75% of the mRNAs examined had A in that position, and another 20% had G. Some experimental evidence for the importance of A or G in position -3, and G in position +4 (immediately following the AUG codon), has been obtained by measuring the binding of synthetic oligonucleotides to wheat germ ribosomes *in vitro* (Kozak, 1981). By applying site-directed mutagenesis to a cloned preproinsulin gene, point mutations were created near the AUG initiator codon; translation of those mutants *in vivo* confirmed the requirement for a purine in position -3 (Kozak, 1984b). Using a more efficient scheme for mutagenesis, I have now obtained a larger set of mutants. Base substitutions in at least four positions near the AUG codon modulate its function, as described below. The sequence ACCAUGG emerges as the best context for initiation in this system. Moré et al. (1985) recently described an  $\alpha$ -thalassemia in which the sequence at the initiation site for  $\alpha$ -globin was changed from CACCAUG to CCCCAUG. The resulting deficiency in globin synthesis confirms, in a natural setting, the importance of A in position -3.

There are a few eukaryotic mRNAs in which an AUG codon in an excellent context (such that all 40S subunits should initiate exclusively there) occurs upstream of a second AUG codon which is known to function. The scanning mechanism as outlined above cannot explain that anomaly. Initiation at the internal AUG triplet in such messages has been attributed to reinitiation, which seems reasonable because access to the downstream cistron is critically dependent on having a terminator codon in frame with the first AUG codon and upstream from the second (Dixon and Hohn, 1984; Hughes et al., 1984; Kozak, 1984c; Liu et al., 1984). Our interpretation is that the 40S ribosomal subunit remains on the mRNA at the terminator codon and resumes scanning; it stops and reinitiates when it reaches the next (internal) AUG codon. The similarity between primary initiation (i.e. selection of the 5'-proximal AUG codon) and reinitiation is emphasized by the finding, described herein, that the optimal context for the AUG codon is the same for both processes.

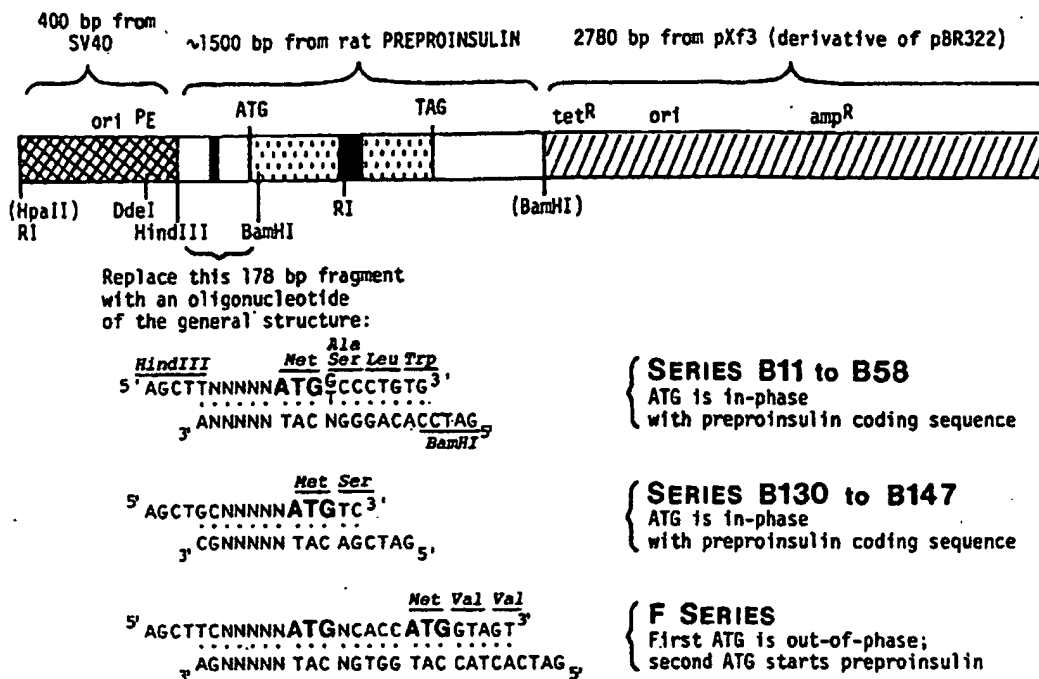


Figure 1. Scheme for Generating Point Mutations around the ATG Codon in Plasmids That Express Preproinsulin

The parental plasmid p255/11 is shown in linear form at the top. Transcription of the rat preproinsulin gene is mediated by the SV40 early promoter, designated P<sub>E</sub>. Shaded areas represent introns that interrupt the 5'-noncoding sequence (open areas) and the preproinsulin coding sequence (stippled). The Bam HI site shown in parentheses is present in the original p255 (Lomedico and McAndrew, 1982) but was eliminated from p255/11 (see Experimental Procedures). "N" indicates positions where the sequence of the oligonucleotides was ambiguous, generating mutations around the ATG codon. The general form of the mutagenic insertion sequences is shown here; the specific oligonucleotides used in the experiments are listed in Table 1. In the B series, mutations were introduced around an ATG codon that starts the preproinsulin coding sequence. In the F series, the focus of mutagenesis was an upstream, out-of-frame ATG codon that serves as a barrier to initiating preproinsulin. The N-terminal amino acid sequence is shown for the variant form of preproinsulin encoded in each series. In the case of B11 through B58, the encoded protein is identical with wild-type rat preproinsulin (MetAlaLeuTrp).

## Results

### Mutagenesis around the Initiator Codon in Preproinsulin Expression Vectors

Figure 1 shows the scheme used to produce mutants in the B series, in which the sequence flanking the initiator codon was systematically varied. The technique involves deleting the DNA fragment that carries the normal translational start site for preproinsulin, and replacing it with a synthetic ATG-containing oligonucleotide: the ATG codon carried on the insert functions as the new initiation site for preproinsulin. (Since all manipulations in this study were at the DNA level, I shall refer to the initiator codon as ATG irrespective of whether the reference is to DNA or mRNA.) The presence of sticky ends on the oligonucleotide that are complementary to those on the acceptor DNA ensures efficient insertion, and the presence of sequence ambiguities within the oligonucleotide generates a large number of mutants. Matteucci and Heynecker (1983) were the first to use a technique similar to this for analyzing translation in *E. coli*.

Multiple insertions were precluded by carrying out the ligation reaction without prior 5' phosphorylation of the oligonucleotide; thus, each recovered mutant carried a single copy of the oligonucleotide. Only when the mismatch occurred very close to the end of the oligonucleotide, in the penultimate or antepenultimate position, did I encounter difficulty in obtaining the nearly complete set of mutants expected. Occasionally, however, one member of a series was not found, despite the repeated isolation of other members of the set; in such cases, a new oligonucleotide was prepared that encoded unambiguously the desired sequence. Mutants in which the sequence of the insert did not correspond precisely to the starting oligonucleotide were recovered infrequently; when found, they usually deviated in one position from the input oligonucleotide. No mutations were found outside of the region encompassed by the oligonucleotide insert.

The screening of mutants was facilitated by varying only two or three positions at a time. Thus, three oligonucleotides, each of which conforms to the general structure shown in Figure 1, were actually used to obtain mutants



Table 1. Oligonucleotides Used for Insertion Mutagenesis

Mutants	Oligonucleotides
B11 - B21	AGCTTCCANNATGCCCCGTG
B31 - B39	AGCTTGGATTATGCCCCGTG
B41 - B58	AGCTTGGANNATGCCCCGTG
B130 - B135	AGCTGCCAAACATGTC
B137 - B138	AGCTGCTTATTATGTC
B140 - B143	AGCTGCTATTATGTC
B145 - B147	AGCTGCTTTTATGTC
F1 - F8	AGCTTCTGATTATGACCATGCTAGT
F9 - F10	AGCTTCTGATTATGACCATGCTAGT
F11 - F15	AGCTTCCATTATGACCATGCTAGT

Sequences are listed for the top (plus) strand of each duplex oligonucleotide that was inserted between the Hind III and Bam HI sites of p255/11. "N" stands for a mixture of all four nucleotides. The mutants that were obtained are listed in the left column; their general form is depicted in Figure 1, and the precise sequence around the ATG codon in each mutant is given in Figures 2 through 6.

B11 through B58. The sequences of the specific oligonucleotides used for mutagenesis are given in Table 1. In the case of B11 through B58, the protein initiated at the inserted ATG codon has the same N-terminal amino acid sequence as wild-type preproinsulin. For reasons of economy, shorter oligonucleotides were used to obtain mutants B130 through B147, and the first few amino acids of the wild-type protein were not retained. This does not compromise the results that follow because the yield of proinsulin from each mutant was always compared, not to the wild-type plasmid, but to a control from the same series as the mutant.

To determine how single base changes around the initiator codon affect translational efficiency, the mutant plasmids were transfected into monkey (COS) cells as described in Experimental Procedures. Two days after transfection, the cells were incubated with <sup>35</sup>S-cysteine, and labeled proteins were extracted, immunoprecipitated, and analyzed by polyacrylamide gel electrophoresis. Because the primary translation product undergoes cleavage in these cells, the product that accumulates and was measured is proinsulin. Measurement of cytoplasmic RNA levels, as described in Experimental Procedures, revealed no significant differences among the mutants in a given series. Thus, the observed variation in proinsulin synthesis reflects the efficiency with which the mRNA produced by each plasmid is translated.

#### Effects of Mutating Positions -3 and +4

The sequences of mutants B31 through B39 are identical except for positions -3 and +4. Single nucleotide changes in those positions modulate the yield of proinsulin over a 20-fold range (Figure 2). Comparison of B35, B38, and B39 shows that A functions better than G, and G better than T, in position -3. Comparison of B38 with B34, or B39 with B33, reveals that G works better than T in position +4. The contributions of positions -3 and +4

are not simply additive. For example, G in position +4 enhanced translation about 5-fold with T in position -3 (B34 versus B38), 4-fold with G in position -3 (B33 versus B39), and only 2-fold with A in position -3 (B31 versus B35). Similarly, with mutants B31 through B34, where the favored nucleotide G occurs in position +4, the effects of varying position -3 were less dramatic than in mutants that had T in position +4. The hierarchy in position -3 (A > G > T) does not change, but the magnitude of the effect obtained upon mutating position -3 depends on how favorable the rest of the sequence is.

The data in Figure 2 confirm that proinsulin is quantitatively recovered in the first round of immunoprecipitation. The second cycle is not shown in the figures that follow.

#### Effects of Mutating Positions -1, -2, -4, and -5

Mutagenesis was limited to positions -1 and -2 in the first experiment. As shown in Figure 3, C in both of those positions enhanced translation marginally, at best. The bracketed lanes in that figure represent duplicate plates that were transfected with the same plasmid, establishing that the variability of the assay is <20%. With that in mind, it seems safe to draw conclusions about plasmids that differ 2-fold or more in their production of proinsulin; but when the increment is less than 2-fold, as with B41, the result cannot be considered more than suggestive. The conclusion from Figure 3 is that, if the nucleotides in positions -1 and -2 influence translation at all, their contributions are small.

To pursue the issue, I constructed the mutants shown in Figure 4. The 3-fold decrease in the yield of proinsulin between B137 and B138 confirms that A works better than C in position -3, as we already knew. The 3-fold increase in proinsulin between B138 and B130 provided the first evidence that C in (some or all of) positions -1, -2, -4, and -5 enhances translation. The effect can also be seen with mutants that have A in position -3 (B137 versus B133), although the stimulation was only 2-fold. (As noted above, nonadditivity can minimize the effects of some sequence changes. Indeed, introducing C into positions -1, -2, -4, and -5 was without measurable effect in mutants that had the optimal A in position -3 plus G in position +4 [B11 through B21, data not shown].) The mutants studied in Figure 4 provide two peripheral insights: C in position -3 apparently functions better than T (B138 versus B140); and, in mutants that lack A in position -3, the presence of A in position -2 or -4 does not compensate. Whereas A in position -3 stimulates translation about 10-fold (B137 versus B140), mutants B141 and B143 translate only marginally better than B140.

Since the stimulatory effect of Cs in positions -1, -2, -4, and -5 was more apparent in plasmids that had C rather than A in position -3 (Figure 4), and since T<sub>-3</sub> seemed to be even weaker than C<sub>-3</sub>, it seemed that plasmids with T in position -3 might provide the most sensitive background for further studies. Accordingly, the mutants shown in Figure 5 were constructed and analyzed. Introducing Cs into positions -1 and -2 indeed stimulated translation at least 4-fold (B145 versus B140). Surprisingly, however, Cs in positions -4 and -5 did not

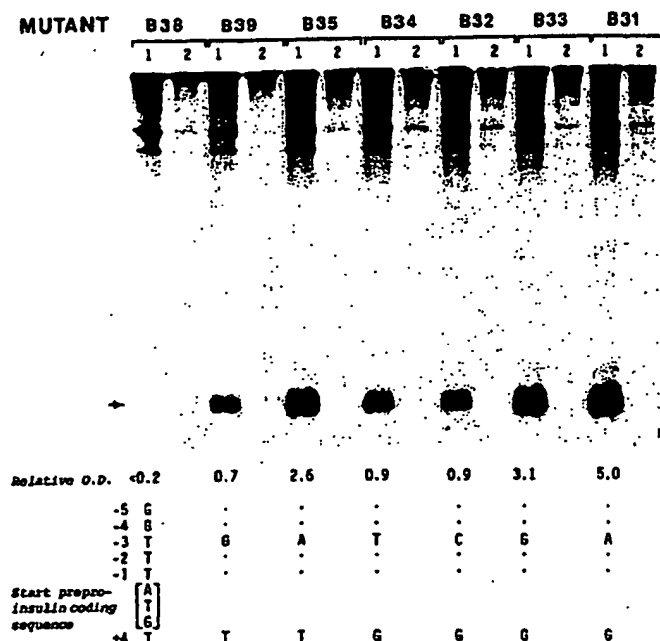


Figure 2. Variation in Proinsulin Synthesis Caused by Single Base Changes in Positions -3 and +4

<sup>35</sup>S-labeled proteins from transfected COS cells were subjected to two rounds of immunoprecipitation which were analyzed in contiguous lanes of the gel, marked 1 and 2. An arrow indicates the position of proinsulin. To make the figure easily readable, only the nucleotides that differ among members of the set are indicated. The sequence shown for B38 in positions -1, -2, -4, and -5 is common to the whole set.

enhance translation (B146 versus B140). The yield of proinsulin from B147, where Cs occur in all four positions, was no greater than from B145.

The 20-fold variation in proinsulin synthesis among mutants in the B series confirms that sequences flanking the ATG codon modulate translational efficiency, but the B mutants do not reveal what happens at a weak ATG codon. The scanning model predicts that some 40S subunits bypass an ATG codon that occurs in an unfavorable context, and translation begins farther downstream. To test that prediction, mutants were needed in which initiation at a downstream ATG codon could be monitored. The F series described below meets that requirement.

#### Effects of Single Base Substitutions around an Upstream ATG Codon

The general form of the oligonucleotides that were inserted to generate mutants in the F series is shown in Figure 1. The position of the second ATG triplet carried on the insert enables it to serve as the initiator codon for preproinsulin, and the sequence around that ATG codon was not mutated. Rather, point mutations were introduced around an upstream, out-of-frame ATG triplet that was expected to function as a "barrier," reducing the number of 40S ribosomes that reach the preproinsulin start site. For this design to work, it was important that no terminator codons occur between the upstream ATG triplet and the preproinsulin start site: eukaryotic ribosomes can reinitiate at the second ATG codon when an upstream "minicistron" terminates prior to the second ATG codon (see Introduction), and the inhibitory effect of an upstream ATG

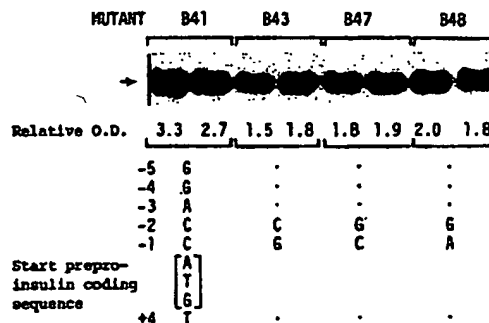


Figure 3. Proinsulin Synthesis by Plasmids That Vary in Positions -1 and -2

Adjacent lanes enclosed by brackets in this and subsequent figures represent <sup>35</sup>S-labeled proteins from duplicate plates that were transfected with the same plasmid. The fluorograms have been cropped to show only the proinsulin region of the gel, which is marked by an arrow. The sequence that is shown for B41 in positions -3, -4, -5, and +4 is common to all four plasmids used for this experiment.

barrier is thereby reversed. To preclude reinitiation in the F series, the reading frame established by the upstream ATG codon had to extend beyond the preproinsulin start site; but the ideal site for termination was hard to determine. When the upstream cistron is long, i.e. when it overlaps the preproinsulin coding sequence over a considerable distance, there is interference (I think at the level of elongation) such that the yield of proinsulin is low even when the upstream ATG triplet occurs in a weak context



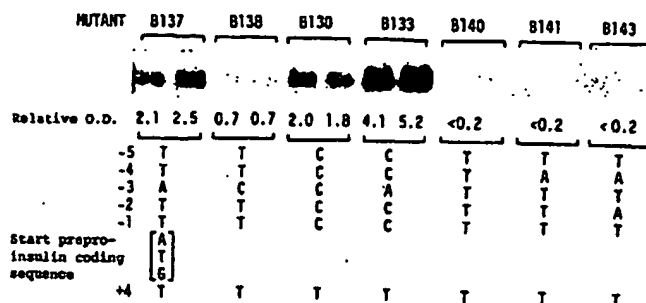


Figure 4. Expanded Mutagenesis of Sequences Preceding the ATG Codon  
Details are given in the legend to Figure 3.

(Kozak, 1984c). Since that unexplained interference decreases as the extent of overlap between the two cistrons decreases, it might seem ideal for the codon that terminates the upstream cistron to overlap the ATG codon that initiates preproinsulin. Unfortunately, that overlapping configuration still allows reinitiation, albeit very inefficiently. The sequence ATGTAG, which I ultimately chose for the F series, allows about 2% reinitiation (based on unpublished experiments with other mutants). It was a workable compromise: the potential sources of interference outlined above were sufficiently reduced that I could see systematic effects of context on the function of upstream ATG codons.

The mutants listed in Figure 6 were studied to determine how single base substitutions around the first ATG codon affect the ability of ribosomes to reach the second, where preproinsulin initiates. F10, which lacks an upstream ATG triplet, is the control to which all other F mutants are compared. In F9, the upstream ATG codon lies in a very weak context and, as expected, synthesis of proinsulin was only slightly reduced. As the context around the upstream ATG codon improves, it becomes a more effective barrier: synthesis of proinsulin dropped 5-fold with F6, F7, and F8, and 10-fold with F1 through F5. It is not surprising that F1—which has the optimal A in position -3 and G in position +4—still makes a trace of proinsulin. As shown in experiment 2 in Figure 6, the upstream barrier can be further strengthened by introducing Cs into the flanking positions. Moreover, because the structure of the F mutants allows a low level of reinitiation, synthesis of proinsulin cannot be shut off completely.

Whereas the results obtained with the B series allow one to conclude only that an ATG codon in one context works better than another, the results obtained with the F series justify the interpretation that 40S ribosomal subunits bypass an ATG codon that lies in a weak context.

#### Context Effects on Reinitiation

A 65 bp sequence that carries an ATG initiator codon, followed after 12 bp by a TAA terminator codon, was inserted at the Hind III site that lies just upstream of the preproinsulin start site in B34, B35, B39, and B38. Thus, ribosomes can make preproinsulin only by reinitiating at the second ATG codon in mutants B34R, B35R, B39R, and B38R. The yield of proinsulin from those plasmids varied about

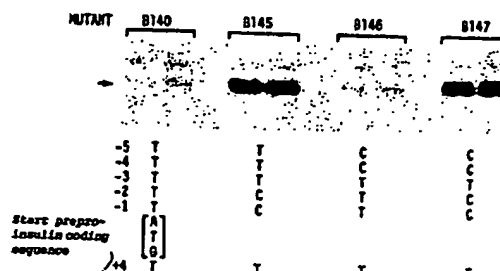


Figure 5. Translation is Stimulated by C in Positions -1 and -2 When the Rest of the Sequence is Suboptimal  
Details are given in the legend to Figure 3.

8-fold, as shown in Figure 7. The "R" derivatives showed the same strong preference for a purine in position -3, and G in position +4, as was seen with the original B mutants. The control B37R lacks the second ATG codon and makes no proinsulin (Figure 7, bottom lane). This rules out the possibility that the proinsulin produced by the other "R" derivatives is actually initiated at the ATG codon carried on the insert (which would require suppression of the terminator codon and posttranslational cleavage to remove the N-terminal amino acid extension) rather than by reinitiating at the ATG codon that directly precedes the preproinsulin coding sequence.

#### Discussion

##### The Optimal Sequence for Initiation in Eukaryotes

From the foregoing mutational analysis, the sequence ACCATGG emerges as the most favorable context for initiation. Although I obtained no evidence that Cs in positions -4 and -5 are part of the ribosome recognition sequence, there is a lingering possibility that Cs in those positions contribute, but only in a small way, and perhaps only when a purine occurs in position -3. Inasmuch as Cs in those positions are highly conserved, I shall continue to show them, in parentheses, as part of the consensus sequence.

The validity of these experiments was ensured in several ways. In many experiments duplicate plates of COS cells were transfected with a given plasmid; the vari-



Figure 6. Effects of Context on the Efficiency of an Upstream ATG Barrier

The sequence shown for F1 in positions -1, -2, -4, and -5 is common to mutants F1 through F10. All have an upstream ATG codon, except for the control F10. The fluorograms have been cropped, and an arrow marks the position of proinsulin.

ation between duplicates never exceeded 20%. When too many plasmids had to be tested simultaneously, I did not assay in duplicate because it is hard to maintain precision when handling more than 12 or 14 plates; but subsequent repetitions of the experiment gave identical results. I considered one plasmid to be translated more efficiently than another only when the yield of proinsulin differed by at least 2-fold; often the difference was 5 to 20 fold. Every position in the consensus sequence has been tested in two or more independent constructs. For example, the combination of T in positions -3 and +4 was shown to be extremely weak in B38 and B140. Adenosine in position -3 worked best in B31, B35, B133, and B137. Comparison of B34 with B38, B33 with B39, and B31 with B35 confirms that G worked best in position +4. Comparison of B130 with B138, B133 with B137, and B147 with B140 confirms that C in some or all of positions -1, -2, -4, and -5 worked better than T. Finally, the results of the F series complement the B series: the sequence that gave the lowest yield of proinsulin when tested directly ( $T_{-3}/T_{+4}$  in B38) allowed the maximum production of proinsulin when that sequence was introduced as an upstream "barrier" in mutant F9.

Although the importance of A or G in position -3 was recognized easily and early (Kozak, 1984b), it was more difficult to show that other nearby nucleotides are recognized, because their contributions are not additive. The nonadditivity was an unwelcome experimental complication, but it has a positive aspect in that most natural ribosome binding sites are buffered: given a purine in position -3, a mutation in position -1, -2, -4, or -5 should reduce translation only slightly. Thus, it is not surprising that mutations in those positions have not been described among the genetic diseases that have been studied at the

molecular level. The dominant effect of position -3 in eukaryotic ribosome binding sites differs from the prokaryotic Shine-Dalgarno sequence, within which no single position is more important than any other. In striking contrast with the enhanced translation that occurred when A was introduced into position -3, there was little stimulation when A occurred in position -2 or -4 (compare B140 with B143 in Figure 4). Thus, if two separate components on the ribosome are responsible for recognizing the upstream A residue and the ATG codon, respectively, the two components must be rigidly oriented. This inflexibility with respect to the position of the upstream recognition sequence again differs from prokaryotes, where the distance between the initiator codon and the Shine-Dalgarno sequence is permitted to vary over a limited range (Kozak, 1983a).

The optimal context for recognition of the ATG codon appears to be the same for reinitiation at an internal site as for primary initiation at the 5'-proximal ATG codon. Our understanding of reinitiation is admittedly primitive, but a likely scenario is that, when an 80S ribosome reaches a terminator codon (having just translated a cistron or minicistron near the 5' end of the mRNA), the 60S subunit detaches while the 40S subunit remains bound to the message and resumes scanning; when the 40S subunit reaches the next ATG codon, it reinitiates translation. An alternative mechanism postulates that ribosomes can bind directly to the internal site. Evidence against direct binding has been adduced previously (Kozak, 1984c). The notion of direct binding might have been revived had the present study revealed an optimal context for internal initiation different from the  $A_{-3}/G_{+4}$  motif that mediates recognition of the 5'-proximal ATG codon. Since the optimal context is the same for both processes, however, it seems

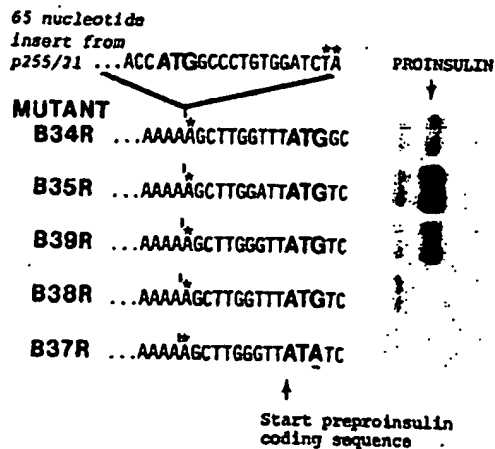


Figure 7. Single Nucleotide Changes in Positions -3 and +4 Affect the Efficiency of Reinitiation

In these "F" derivatives, ribosomes first initiate at the invariant ATG codon carried on the insert, terminate at the TAA codon (marked by asterisks), and then reinitiate 10 nucleotides downstream, at the start of the preproinsulin coding sequence, which differs (see positions -3 and +4) among the four mutants tested. B37R is a negative control described in the text. <sup>35</sup>S-labeled proteins from transfected COS cells were analyzed by polyacrylamide gel electrophoresis. The top of the gel (not shown) was at the left.

likely that reinitiation involves a replay of the scanning process.

#### Secondary Structure Does Not Contribute to the Observed Variation in Translation

Although secondary structure might differ slightly from one B mutant to another, that is unlikely to have influenced the results of this study. In an experiment described elsewhere (Kozak, 1986), I deliberately created a stable hairpin ( $\Delta G = -30 \text{ kcal/mol}$ ) around the ATG codon in a mutant called B13hp; the hairpin did not reduce the yield of proinsulin. Thus 40S ribosomal subunits and/or the associated initiation factors have an impressive ability to melt duplex structures in mRNA. Since the point mutations described herein cannot create duplexes anywhere near as stable as the hairpin in B13hp, the 20-fold variation in proinsulin yield among members of the B series is best interpreted at the primary sequence level. The secondary structure hypothesis is also inconsistent with the dramatic inhibition that occurs upon changing a single nucleotide in position -3, compared with the small change in translation when several nearby nucleotides are mutated.

#### Incorporation of Context Rules into the Scanning Model

Our working hypothesis is that the migration of 40S subunits is halted more or less efficiently depending on the sequence around the ATG codon. This rationalizes the results obtained with the F series, where the strength of the upstream ATG "barrier" varies in the predicted way with changes in context. Constructs have been described previously in which the presence of an upstream ATG

codon reduces or supplants initiation from the downstream site (Lomedico and McAndrew, 1982; Smith et al., 1983; Krieg et al., 1984) and, in some cases, the efficiency of the upstream ATG barrier was shown to be context dependent (Bandyopadhyay and Tamin, 1984; Kozak, 1984c; Liu et al., 1984). The rules deduced by manipulating such cloned genes apparently hold for natural mRNAs as well—specifically for a number of viral mRNAs that have the unusual ability to produce two proteins. The first ATG codon in such mRNAs is usually in a weak context, thus rationalizing the ability of some ribosomes to reach the second ATG codon. There are at least ten examples from animal virus systems of bifunctional mRNAs that fit this pattern (Reddy et al., 1978; Bos et al., 1981; Clerx-van Haaster et al., 1982; Giorgi et al., 1983; Heermann et al., 1984; Laprevotte et al., 1984; Bellini et al., 1985; Castle et al., 1985; Clarke et al., 1985; Ernst and Shatkin, 1985; Persing et al., 1985; Sarkar et al., 1985). Such mRNAs in which there are two prominent initiation sites are rare. In most eukaryotic mRNAs the 5'-proximal ATG codon lies in a fairly strong context and ribosomes initiate predominantly at that site. To make more precise statements, we have to recognize that initiation sites are not simply strong or weak; the mutants described herein reveal a gradient of strength. The sequence (CC)ACCATGG, ranks highest in efficiency: when that sequence occurs near the 5' end of a message, no initiation can be detected downstream (see Kozak, 1983b; 1984c [mutants p255/20 and C2]; and mutant E13 in Kozak, 1986). A sequence such as ATTATGT, on the other hand, is also very efficient if one simply monitors the yield of protein initiated at that site (B35 in Figure 2). But in mutant F4, ~10% of the ribosomes bypassed the 5'-proximal ATTATGT and initiated at the preproinsulin start site downstream. If we now consider the sequences of natural eukaryotic mRNAs, although ~75% have A in position -3, only ~35% have A<sub>-3</sub> plus G<sub>+4</sub>; and less than 5% have the ideal (CC)ACCATGG sequence. Thus, unless other features can compensate for a less than perfect context around the ATG codon, we should expect to find a second initiation site functioning (albeit very inefficiently) in most eukaryotic mRNAs. I suspect that compensatory mechanisms will be discovered. In some mRNAs where the coding sequence begins with a weak ATG codon, the objective might be not so much to allow ribosomes access to a second start site, but simply to limit the synthesis of a protein that would be harmful if overproduced. In the case of preproinsulin mRNA, the functional initiator codon is flanked by Ts in positions -3 and +4, and a strong out-of-frame ATG codon lies just upstream (Lamb et al., 1985). It would be hard to design a less favorable arrangement for translation—or a more toxic polypeptide.

#### By What Mechanism Do Nucleotides Flanking the ATG Codon Exert Their Effect?

Despite many differences between the prokaryotic and eukaryotic initiation mechanisms, the temptation to search 18S rRNA for a sequence that could do for eukaryotes what the Shine-Dalgarno sequence in 16S rRNA does for prokaryotes is irresistible. Figure 8 shows two sites in 18S rRNA that might pair with the (CC)ACC se-

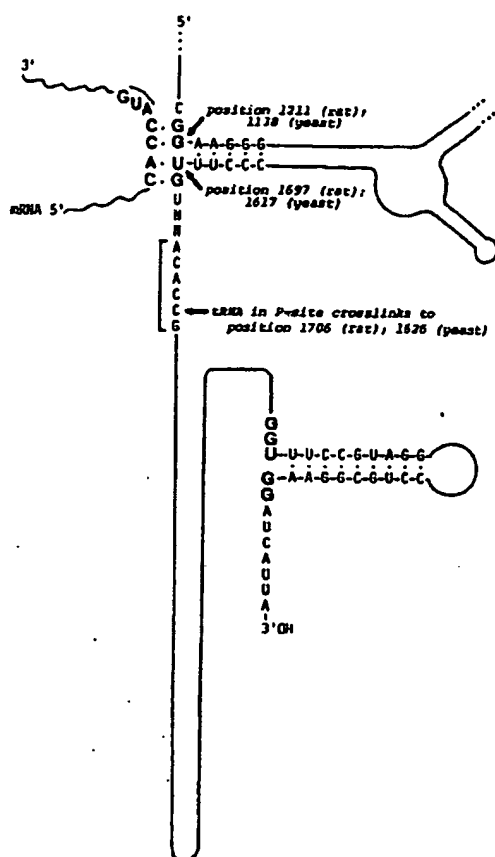


Figure 8. Possible interaction between mRNA and 18S Ribosomal RNA.

Highlighted are two sequences in 18S rRNA that are complementary to the conserved (CC)ACC motif in eukaryotic mRNAs. The GG/UGG sequence that extends across the base of the 3'-proximal hairpin in 18S rRNA was noted previously by Sargan et al. (1982). A novel interaction between mRNA and rRNA is shown close to the P site, where Initiator Met-tRNA is believed to bind. The entire sequence of rat 18S rRNA, from which this segment was redrawn, is given in Chan et al. (1984).

quence in mRNA. Sargan et al. (1982) previously proposed that a noncontiguous GG/UGG sequence, brought together by the conserved hairpin near the 3' end of 18S rRNA, might pair with CCACC in mRNA. Their suggestion that the CCACC motif might function irrespective of its distance from the ATG codon now seems unlikely, but the site that they proposed in 18S rRNA remains interesting. The sequence 3'-GU/UG-5' occurs at the base of another hairpin in the interior of 18S rRNA, just a few nucleotides from the position to which peptidyl-tRNA becomes cross-linked when it is bound in the P site (Ehresmann et al., 1984). Initiator Met-tRNA also binds in the P site, of course. One could elaborate the model by suggesting that the nearby sequence ACACCG (bracketed in Figure 8), by virtue of being complementary to the proposed binding site for

mRNA, might mediate a conformational switch within the rRNA that displaces the mRNA. In a similar vein, the region of *E. coli* 16S rRNA that includes the Shine-Dalgarno site is believed to undergo a rearrangement that ruptures the pairing with mRNA (Yuan et al., 1979). Nakashima et al. (1980) showed that eukaryotic mRNAs in 40S or 80S initiation complexes could be cross-linked by psoralen to 18S rRNA. Although they were specifically looking for an interaction between the 3' end of 18S rRNA and the cap-adjacent sequence in mRNA, their data actually fit better with an interaction between internal sequences in both RNAs.

The conservation of G in position +4 became evident several years ago, when the first few eukaryotic ribosome binding sites were sequenced. The suggestion (Kozak and Shatkin, 1977) that AUGG in mRNA might form a 4 base pair interaction with CCAU in the anticodon loop of initiator Met-tRNA has not yet been tested.

#### Experimental Procedures

##### Construction and Characterization of Mutants in the B and F Series

The parental plasmid p255/11 was derived previously (Kozak, 1984b) from Lomedico's original p255 (Lomedico and McAndrew, 1982) by deleting a Bam HI site at the junction between pBR322 and the rat genomic sequence. p255/11 retains a single Bam HI site, 8 bp down from the ATG initiator codon (Figure 1). After digesting p255/11 with Hind III and Bam HI, the large, linear fragment—which lacks only the four N-terminal amino acids of the preproinsulin coding sequence—was purified by agarose gel electrophoresis. This fragment was used as acceptor in DNA ligase reactions with various synthetic oligonucleotides, which were purchased from Pharmacia L-L Biochemicals. Each of the oligonucleotides listed in Table 1 was annealed with another oligonucleotide that was partly complementary, resulting in duplex structures with single-stranded termini complementary to the Hind III and Bam HI ends of the acceptor DNA. This is illustrated in Figure 1. In preparation for ligation, the oligonucleotides were preannealed but were not phosphorylated. The DNA ligase reaction typically contained 1 µg of linearized acceptor DNA and 0.2  $A_{260}$  units of oligonucleotide in 25 µl. The mixture was incubated at 16°C for 20 hr, heated for 10 min at 65°C to inactivate the enzyme, and used directly to transform *E. coli* as described previously (Kozak, 1983b). DNA from ampicillin-resistant colonies was screened for the desired mutation by direct sequencing of plasmid DNA extracted rapidly from 10 ml cultures (Kozak, 1983b); the sequences were confirmed at a later step using pure DNA. For sequence analysis, DNA was labeled with  $\alpha$ -<sup>32</sup>P-CTP at the Dde I site that lies in the SV40 portion of the leader (Figure 1 in Kozak, 1984b), recut with Eco RI, and subjected to chemical cleavage (Maxam and Gilbert, 1980).

##### Construction of Reinitiation Derivatives

Several plasmids were constructed in which the ability to make proinsulin depends on reinitiating downstream from a terminator codon. The upstream "mimicistron" (i.e., a fragment that contains an ATG initiator codon followed shortly by an in-phase terminator codon) was carried on a Hind III fragment from p255/21, which has been described previously (Kozak, 1984c). DNA from mutants B34, B35, B37 (a control), B38, and B39 was linearized by digesting with Hind III, treated with alkaline phosphatase, and the small 184 bp Hind III fragment from p255/21 was then inserted. This fragment includes a 119 bp intron; thus, the insert in mature mRNA is 65 nucleotides. The structures of the resulting "R" derivatives are shown in Figure 7. The number and orientation of inserts was determined by analysis with appropriate restriction enzymes.

##### Analysis of Proteins and RNA from Transfected COS Cells

COS-1 cells (Gluzman, 1981) in 60 mm plates were transfected one day after plating, when the monolayers were about 75% confluent. Each

plate was transfected with 0.5 ml of calcium phosphate mixture (Wigler et al., 1978) containing 20 µg of purified plasmid DNA and 40 µg of calf thymus carrier DNA. Other details of the procedure were as described previously (Kozak, 1983b). Forty-eight hours after transfection, the cells were rinsed twice with cysteine-free medium and incubated for 4 hr with 1 ml of medium containing 0.25 mCi of <sup>35</sup>S-cysteine (New England Nuclear). The cells were scraped with a rubber policeman into phosphate-buffered saline, and were then lysed with 0.9% NP40 and 0.4% deoxycholate. A 200 µl aliquot of the cytoplasmic extract, representing about 4 × 10<sup>5</sup> cells, was incubated at 4°C for 20 hr with 3 µl of antiserum against bovine insulin (Miles). The immunoprecipitates were recovered using Pansorbin (Calbiochem) and were analyzed by electrophoresis in polyacrylamide gels containing urea and SDS, as described (Kozak, 1983b). The gels were impregnated with Enhance (New England Nuclear), dried, and placed in contact with XAR-5 film for 2 to 20 days at -70°C. The data were quantified by densitometric scanning of the films.

Cytoplasmic RNA levels were analyzed by dot blot hybridizations using a <sup>32</sup>P-labeled riboprobe. The probe was obtained by cloning the 190 bp Bam HI-Eco RI fragment, which represents two-thirds of the coding sequence for rat preproinsulin, into the polylinker region of pSP65. The construct was linearized with Bam HI and transcribed with SP6 polymerase (New England Biolabs), according to Melton et al. (1984). The size of the transcript was checked by electrophoresis on 8% polyacrylamide gels containing 8 M urea. Cytoplasmic RNA from 48 hr transfected COS cells was extracted with phenol, serially diluted, and bound to Gene Screen fibers (New England Nuclear). Prehybridization and hybridization were carried out using the conditions recommended by Melton et al. (1984).

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#### References

- Bandyopadhyay, P. K., and Termin, H. M. (1984). Expression from an internal AUG codon of herpes simplex thymidine kinase gene inserted in a retrovirus vector. *Mol. Cell. Biol.* 4, 743-748.
- Bellini, W. J., Englund, G., Rozenblatt, S., Amthor, H., and Richardson, C. D. (1985). Measles virus P gene codes for two proteins. *J. Virol.* 53, 908-919.
- Bos, J. L., Polder, L. J., Bernard, R., Schrier, P., van den Elsen, P., van der Eb, A., and van Ormondt, H. (1981). The 2.2 kb E1b mRNA of human Ad12 and Ad5 codes for two tumor antigens starting at different AUG triplets. *Cell* 27, 121-131.
- Castle, E., Nowak, T., Leidner, U., Wengler, G., and Wengler, G. (1985). Sequence analysis of the viral core protein and the membrane-associated proteins of flavivirus West Nile Virus. *Virology*, in press.
- Chan, Y.-L., Gutell, R., Noller, H. F., and Wool, I. G. (1984). The nucleotide sequence of a rat 18S ribosomal RNA gene and a proposal for the secondary structure of 18S rRNA. *J. Biol. Chem.* 259, 224-230.
- Clarke, B. E., Sangar, D. V., Burroughs, J. N., Newton, S. E., Carroll, A. R., and Rowlands, D. J. (1985). Two initiation sites for foot and mouth disease virus polypeptide in vivo. *J. Gen. Virol.* 66, 227-238.
- Clen-va Haastert, C., Akashi, M., Auperin, D., and Bishop, D. (1982). Nucleotide sequence analysis and predicted coding of bunyavirus genome RNA species. *J. Virol.* 47, 119-128.
- Dixon, L., and Hohn, T. (1984). Initiation of translation of the cauliflower mosaic virus genome from a polycistronic mRNA: evidence from deletion mutagenesis. *EMBO J.* 3, 2731-2738.
- Ehresmann, C., Ehresmann, B., Milon, R., Ebel, J.-P., Nuss, K., and Ofengand, J. (1984). Cross-linking of the anticodon of E. coli and E. subtilis acetylaldehyde-tRNA to the ribosomal P site. *Biochemistry* 23, 429-437.
- Ernst, H., and Shatkin, A. J. (1985). Reovirus hemagglutinin mRNA codes for two polypeptides in overlapping reading frames. *Proc. Natl. Acad. Sci. USA* 82, 48-52.
- Giorgi, C., Blumberg, B., and Kolakofsky, D. (1983). Sendai virus contains overlapping genes expressed from a single mRNA. *Cell* 35, 829-838.
- Guzman, Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* 23, 175-182.
- Heermann, K., Goldmann, U., Schwartz, W., Seyffarth, T., Baumgarten, H., and Gerlich, W. H. (1984). Large surface proteins of hepatitis B virus containing the pre-S sequence. *J. Virol.* 52, 396-402.
- Hughes, S., Mellstrom, K., Kosik, E., Tamanoi, F., and Brugge, J. (1984). Mutation of a termination codon affects initiation. *Mol. Cell. Biol.* 4, 1738-1748.
- Konarska, M., Filipowicz, W., Domdey, H., and Gross, H. J. (1981). Binding of ribosomes to linear and circular forms of the 5'-terminal leader fragment of tobacco mosaic virus RNA. *Eur. J. Biochem.* 114, 221-227.
- Kozak, M. (1979). Inability of circular mRNA to attach to eukaryotic ribosomes. *Nature* 280, 62-65.
- Kozak, M. (1980a). Evaluation of the "scanning model" for initiation of protein synthesis in eucaryotes. *Cell* 22, 7-8.
- Kozak, M. (1980b). Role of ATP in binding and migration of 40S ribosomal subunits. *Cell* 22, 459-467.
- Kozak, M. (1981). Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. *Nucl. Acids Res.* 9, 5233-5252.
- Kozak, M. (1983a). Comparison of initiation of protein synthesis in prokaryotes, eucaryotes, and organelles. *Microbiol. Rev.* 47, 1-45.
- Kozak, M. (1983b). Translation of insulin-related polypeptides from mRNAs with tandemly reiterated copies of the ribosome binding site. *Cell* 34, 871-878.
- Kozak, M. (1984a). Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucl. Acids Res.* 12, 857-872.
- Kozak, M. (1984b). Point mutations close to the AUG initiator codon affect the efficiency of translation of rat preproinsulin in vivo. *Nature* 308, 241-248.
- Kozak, M. (1984c). Selection of initiation sites by eucaryotic ribosomes: effect of inserting AUG triplets upstream from the coding sequence for preproinsulin. *Nucl. Acids Res.* 12, 3873-3883.
- Kozak, M. (1985). Influences of mRNA secondary structure on initiation by eucaryotic ribosomes. *Proc. Natl. Acad. Sci. USA*, in press.
- Kozak, M., and Shatkin, A. J. (1977). Sequences and properties of two ribosome binding sites from the small size class of reovirus mRNA. *J. Biol. Chem.* 252, 6895-6908.
- Kozak, M., and Shatkin, A. J. (1978). Migration of 40S ribosomal subunits on mRNA in the presence of edeine. *J. Biol. Chem.* 253, 6568-6577.
- Krieg, P., Strachan, R., Wallis, E., Tabe, L., and Colman, A. (1984). Efficient expression of cloned complementary DNAs for secretory proteins after injection into *Xenopus* oocytes. *J. Mol. Biol.* 180, 615-643.
- Lamb, F. I., Roberts, L. M., and Lord, J. M. (1985). Nucleotide sequence of cloned cDNA coding for preproinsulin. *Eur. J. Biochem.* 148, 265-270.
- Laprevotte, I., Hampe, A., Sherr, C., and Galibert, F. (1984). Nucleotide sequence of the gag gene and gag-pol junction of feline leukemia virus. *J. Virol.* 50, 884-894.
- Li, C.-C., Simonsen, C. C., and Levinson, A. D. (1984). Initiation of translation at internal AUG codons in mammalian cells. *Nature* 309, 82-85.
- Lomedico, P., and McAndrew, S. (1982). Eukaryotic ribosomes can recognize preproinsulin initiation codons irrespective of their position relative to the 5'-end of mRNA. *Nature* 299, 221-228.
- Matteucci, M. D., and Heyneker, H. L. (1983). Targeted random mutagenesis: the use of ambiguously synthesized oligonucleotides to

- mutagenize sequences immediately 5' of an ATG initiation codon. *Nucl. Acids Res.* **11**, 3113-3121.
- Maxam, A. M., and Gilbert, W. (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. *Meth. Enzymol.* **65**, 499-560.
- Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucl. Acids Res.* **12**, 7035-7058.
- Morlé, F., Lopez, B., Hanni, T., and Godet, J. (1985).  $\alpha$ -Thalassemia associated with the deletion of two nucleotides at position -2 and -3 preceding the AUG codon. *EMBO J.* **4**, 1245-1250.
- Nakashima, K., Darzynkiewicz, E., and Shatkin, A. J. (1980). Proximity of mRNA 5'-region and 18S rRNA in eukaryotic initiation complexes. *Nature* **286**, 226-230.
- Persing, D. H., Varmus, H. E., and Ganem, D. (1985). A frameshift mutation in the pre-S region of the human hepatitis B virus genome allows production of surface antigen particles but eliminates binding to polymerized albumin. *Proc. Natl. Acad. Sci. USA* **82**, 3440-3444.
- Reddy, V. B., Dhar, R., and Weissman, S. M. (1978). Nucleotide sequence of the genes for the simian virus 40 proteins VP2 and VP3. *J. Biol. Chem.* **253**, 621-630.
- Sargan, O. R., Gregory, S. P., and Butterworth, P. H. W. (1982). A possible novel interaction between the 3'-end of 18S rRNA and the 5'-leader sequence of many eukaryotic mRNAs. *FEBS Letters* **147**, 133-138.
- Sarkar, G., Peletier, J., Bassel-Duby, R., Jayasuriya, A., Fields, B. N., and Sonenberg, N. (1985). Identification of a new polypeptide coded by reovirus gene  $\sigma 1$ . *J. Virol.* **54**, 720-725.
- Shatkin, A. J. (1976). Capping of eucaryotic mRNAs. *Cell* **9**, 645-653.
- Smith, A. E. (1977). Cryptic initiation sites in eukaryotic virus mRNAs. *Federation of European Biological Societies Symposium* **43**, 37-46.
- Smith, G. E., Summers, M. D., and Fraser, M. J. (1983). Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Molec. Cell. Biol.* **3**, 2156-2165.
- Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. (1978). Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell* **14**, 725-731.
- Yuan, R. C., Steltz, J. A., Moore, P. B., and Crothers, D. M. (1979). The 3' terminus of 18S rRNA: secondary structure and interaction with ribosomal protein S1. *Nucl. Acids Res.* **7**, 2399-2418.

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# Inoculation of plasmids expressing the dengue-2 envelope gene elicit neutralizing antibodies in mice

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*To develop a nucleic acid vaccine against dengue type-2 virus, the PreM and 92% of the envelope (E) genes were cloned into different eukaryotic plasmid expression vectors (pCMVintPolyli and pVR1012). The resultant plasmid constructs (pD2ME and p1012D2ME) properly expressed the truncated E protein in vitro as evidenced by the expected protein size on SDS-PAGE and the ability of the protein to be recognized by monoclonal antibodies directed against conformational epitopes. Three-week-old BALB/c mice were given intradermal inoculations of each construct. Plasmid expression vectors without dengue genes were used as controls. One hundred percent of the mice that received the pD2ME and p1012D2ME constructs developed anti-dengue antibodies. These antibodies were shown to neutralize dengue type-2 virus in vitro. This is the first demonstration of the use of nucleic acid inoculation in the development of potential dengue virus vaccines. © 1997 Published by Elsevier Science Ltd.*

**Keywords:** dengue-2 virus; nucleic acid vaccine; neutralizing antibodies

Dengue (Den) viruses belong to the flavivirus genus of the family Flaviviridae, and consist of four antigenically different serotypes, Den 1-4<sup>1</sup>. The viral genome is composed of a single strand of positive sense RNA that codes for three structural and seven nonstructural proteins<sup>2</sup>.

Dengue viruses are transmitted primarily by the mosquito, *Aedes aegypti*, and are a major cause of morbidity and mortality throughout tropical and subtropical regions worldwide<sup>3</sup>. Annually, it is estimated that there are over 100 million cases of Den fever<sup>4</sup>. Human Den illnesses range from an acute undifferentiated fever to hemorrhagic fever and shock. A primary infection usually causes Den fever. The illness is generally mild and the person apparently acquires a life-long immunity against the serotype of Den virus causing the infection. If a person acquires a second Den infection with a different serotype, the illness is more likely to be severe and lead to hemorrhagic fever or shock syndrome. The increased severity of the secondary infection is believed to be caused by an immune enhancement phenomenon<sup>5</sup>.

Although considerable research has been directed towards the development of a safe, effective Den vaccine over the past 50 years, no approved product is presently available. Early studies on the use of inactivated Den

viruses as vaccines reported that the inactivation process resulted in partial loss of immunogenicity<sup>6</sup>. More recent studies with recombinant protein vaccines have generally been disappointing because of the low levels of neutralizing antibody induced or the lack of protection from subsequent live virus challenge in the subhuman primate model<sup>6,7</sup>. A number of human trials have been conducted with live Den virus vaccines. Early trials using Den viruses attenuated by serial cerebral passage in mice or passage in chick embryos were encouraging, but concerns about the presence of brain tissue and increased neurovirulence led to abandonment of this approach<sup>8</sup>. Most recently, groups have tried serial passage in primary dog kidney cell cultures to select live-attenuated Den viruses with mixed success. Some attenuated vaccine candidates yield high levels of protective immunity, but the presence of clinical complications has made them undesirable for use<sup>8,9</sup>. Another group<sup>10</sup>, has used this approach to obtain an attenuated vaccine which exhibits protective immunity and only minimal clinical symptoms.

We have taken the newly developed nucleic acid vaccination approach to produce a Den virus vaccine candidate. This approach has recently been successfully demonstrated in animal models for other infectious agents including malaria<sup>11</sup>, HIV<sup>12</sup>, influenza<sup>13</sup> and hepatitis C<sup>14</sup>. Nucleic acid vaccines possess many of the advantages of live-attenuated vaccines without the concern of inducing clinical illness by the vaccine itself. Since the viral proteins are translated and processed within the host cells, proper conformation of B cell epitopes on secreted proteins and induction of class I

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MHC-dependent immune responses should occur appropriately. The humoral and cellular immune responses elicited by DNA immunization appear to be long lasting<sup>15</sup>. In this article, we describe the first report of the use of nucleic acid vaccine technology to develop a DNA plasmid construct that elicits anti-Den neutralizing antibodies in a mouse model.

## MATERIALS AND METHODS

### Preparation of plasmid DNA constructs

The plasmid pKT2.4 was kindly provided by Dr R. Putnak, Walter Reed Army Institute of Research, Washington, DC. This plasmid contains the capsid (C), pre-membrane (PreM), and envelope (E) genes of dengue-2 (Den-2), New Guinea C strain. The Den-2 sequences, spanning bases 1-2249 (including C, PreM and 92% of E), were retrieved from this plasmid by EcoRI digestion and polished with PFU polymerase (Statagene Cloning Systems, La Jolla, CA). The polished restriction fragment was then digested with Sal I and cloned into the Sal I/EcoR V sites of pCMVint-Polyli (Vical Inc., San Diego, CA). This resultant plasmid is pCME. To remove C sequences, pCME was digested with Pst I, gel purified and re-ligated. The Pst I digestion removes sequences from the multiple cloning site to Den-2 base 327. The resultant plasmid is pD2ME and contains the PreM signal sequence, PreM and 92% E (bases 327-2249). To confirm the presence of Den genes, pD2ME was sequenced on an ABI 377 automated DNA sequencer using primers P2 (5'-CATTGGA TTTGAAGTGA), P4 (5'-GTCACGATGGAGTG CTCT), P5 (5'-GGATGTTGTTGTTTGGGAT), P9 (5'-CCTCTATACAGTACTTCCTTAGAGTGGC), TK1 (5'-GGTAACCTCCCGTTGCGTTCTG), TK4 (5'-CATGGAAGCCATCACAGACGGC).

To construct p1012D2ME, the Den-2 PreM signal sequence, PreM/92% E fragment in pD2ME was removed by Pst I/Bgl II digestion and ligated into the multiple cloning site of the expression vector pVR1012 (Vical Inc., San Diego, CA). To produce sufficient quantities, all plasmids were transformed into *Escherichia coli* DH5 cells, grown in the presence of kanamycin and purified by alkaline lysis<sup>16</sup>. Following alkaline lysis, the plasmids were double CsCl-ethidium bromide gradient purified<sup>16</sup>, resuspended in phosphate buffered saline (PBS) and stored at -20°C until used.

### In vitro analysis of constructs

The expression of Den-2 virus envelope protein by pD2ME was determined in 293 cells (American Type Culture Collection, Rockville, MD) by immune precipitation. The cells were grown to 25% confluence in 60 mm dishes, and were calcium phosphate transfected with 15 µg of plasmid DNA<sup>16</sup>. The cells were maintained in DMEM with 10% fetal calf serum, Pen/Strep at 37°C in 5% CO<sub>2</sub>. The media was changed 12 h post-transfection. Thirty-six hours post-transfection, the cells were washed with PBS and incubated with 50 µCi [<sup>35</sup>S]methionine (1000 Ci mmol<sup>-1</sup>, ICN) in 2 ml methionine-deficient media for 12 h. The media and the cells were then subject to immunoprecipitation using Den-2 hyperimmune ascitic fluid (HIAF) (American Type Culture Collection, Rockville, MD) and monoclonal antibodies

4G2 and 3H5<sup>17</sup>. Prior to immunoprecipitation, the media was removed and the cells were scraped off the plates and lysed in 1 ml RIPA buffer (0.05 M Tris-HCl, pH 7.5; 0.15 M NaCl; 1% NP-40; 0.5% Deoxycholate; 0.1% SDS). For the immunoprecipitations, 2 ml media or 300 µl cell lysate was mixed with 5 µl antisera and 25 µl protein G sepharose (BRL) and incubated at 4°C for 1 h. The precipitates were collected, washed twice with PBS, resuspended in Laemmli buffer (0.015 M Tris-HCl, pH 6.8; 0.1 M DTT; 2% SDS; 0.1% Bromophenol Blue; 10% glycerol), boiled for 10 min followed by 10% SDS-PAGE<sup>16</sup>. The gel was then fixed for one h with 10% acetic acid, 25% methanol at 25°C, dried and developed by autoradiography.

The expression of both pD2ME and p1012D2ME were evaluated by immunofluorescence assay (IFA). To accomplish this, the plasmids were transfected as above. Forty-eight hours post-transfection the media was removed from the cells, 1 ml PBS was added and the cells were scraped off the plates. The cells were washed twice with PBS and resuspended in 1 ml PBS. Ten microliter aliquots were spotted onto slides, air dried and fixed with acetone. IFA was then performed as previously described<sup>18</sup> using Den-2 HIAF and monoclonal antibodies 3H5 and 4G2. The fluorescence was then rated by three different observers.

### Mice and DNA injections

Three-week-old Balb/c mice (Jackson Labs, Bar Harbor, ME) were inoculated intradermally with 200 µg of plasmid DNA in 50 µl of PBS. Injections were given in the tail, ca 2 cm from the base as previously described<sup>19</sup>. The mice were primed on day 0 and boosted on days 9, 22, and 57. Prior to boosting, blood samples were obtained by the periorbital route. Blood samples were also obtained, 35 and 87 days after the last boost. Sera from these samples were stored at -70°C until used. Following each injection the mice were maintained in American Association for Laboratory Animal Care-approved quarters under pathogen-free conditions.

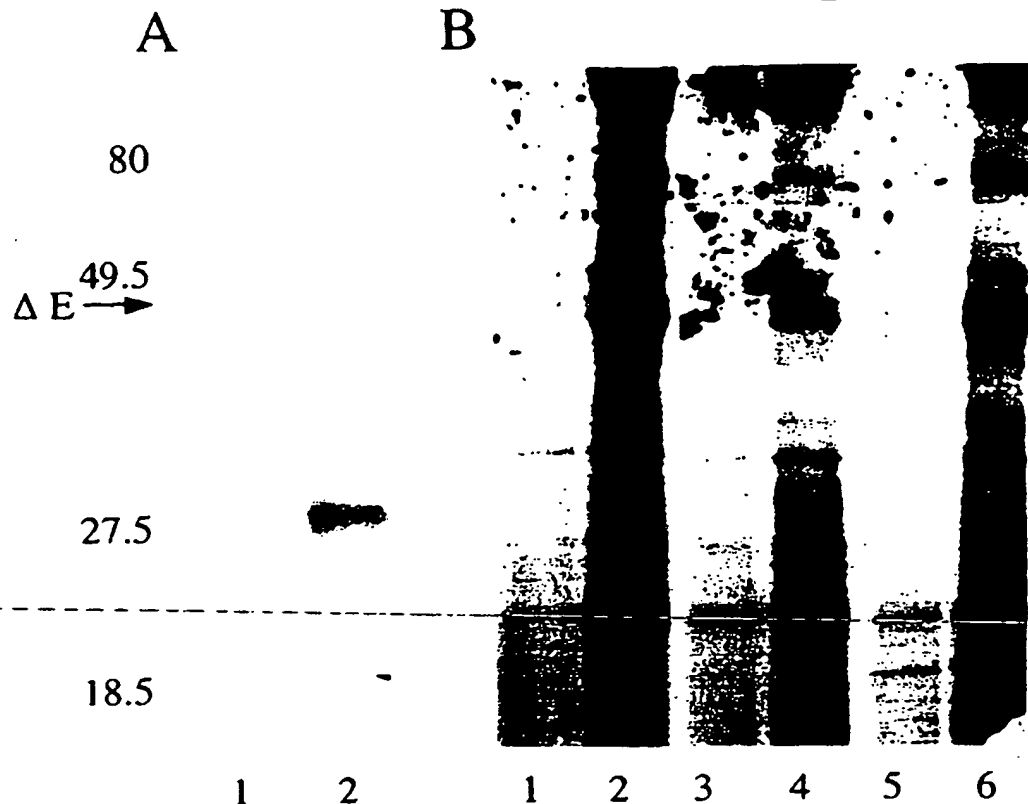
### Den-2 virus challenge of mice

In a parallel study, a second group of mice immunized with the DNA plasmids were challenged with Den-2 virus as described previously<sup>20</sup>. Briefly, three-week-old Balb/c mice were primed intradermally with 200 µg plasmid DNA and boosted with 200 µg DNA 9 days later. At 6 weeks of age the mice were challenged. Challenge virus was prepared from a Den-2 (New Guinea C) infected suckling mouse brain. Mice received 100 mouse 50% lethal doses of Den-2 intracranially. The mice were then monitored for signs of encephalitis and death.

### Detection of mouse anti-Den antibody

Sera were assayed for anti-Den antibodies by enzyme-linked immunosorbent assay (ELISA)<sup>21</sup>. Briefly, Den-2 infected and uninfected Vero cell culture lysates were coated onto 96-well microtiter plates. The serum samples were diluted 1:25 or 1:100 in serum dilution buffer (5% skim milk and 0.1% Tween-20 in PBS), and added in duplicate to the Den-2 antigen coated and uninfected antigen coated plates. After 1 h incubation at 37°C, the plates were washed with PBS and horse radish





**Figure 1** Immune-precipitations of pkCMVintPolyli and pD2ME transfected cells: (A) immune precipitation of media from transfected cells; and (B) immune precipitation of cell lysates. DNA transfected: pkCMVintPolyli (Lanes A1, B1, 3, and 5); pD2ME (Lanes: A2, B2, 4, and 6). Anti-sera: HIAF (lanes: A1,2, B1 and 2); 4G2 (lanes: B3 and 4); 3H5 (lanes: B5 and 6). E→, migration position of truncated E. Molecular weight markers are indicated to the left of the figure

peroxidase conjugated goat anti-mouse IgG, IgM and IgA (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to each well to detect bound mouse anti-Den antibody. The wells were washed and a chromogenic substrate (ABTS) was added and allowed to incubate for 30 min at 37°C. Plates were then read at 410 nm. Adjusted optical density (O.D.) values were calculated by subtracting the O.D. of the uninfected antigen-coated well from the corresponding viral antigen-coated well. The cutoff O.D. value for determining antibody positivity was calculated as the mean adjusted O.D. of the negative control sera plus 3 standard deviations (S.D.).

#### Virus plaque reduction neutralization test (PRNT)

Den-2 New Guinea C virus was propagated in Vero cells, aliquoted and stored as working stock at -80°C until used. The titer of the virus stock was  $2 \times 10^6$  p.f.u. ml<sup>-1</sup>, determined by plaque titration assay in LLC-MK<sub>2</sub> cells<sup>22</sup>. Mouse sera were assayed for Den-2 virus neutralizing antibody in a PRNT as previously described<sup>1</sup>. Percent plaque reduction was calculated for each dilution of test sera using p.f.u. obtained from normal mouse sera at the same dilution as the baseline count. The 50% PRNT titers were determined by probit analysis.

## RESULTS

#### *In vitro* analysis of constructs

The PreM and 92% of E genes of Den-2 New Guinea C strain were cloned into two eukaryotic expression

vectors, pkCMVintPolyli and pVR1012. The resultant plasmid constructs are pD2ME and p1012D2ME, respectively. Sequence analysis revealed that compared to the published Den-2 New Guinea C sequence<sup>23</sup>, pD2ME contains two point mutations and sequences at the 3' end which code for seven nonDen-2 amino acids. The first point mutation is a G to A at base 532 and the second is a C to A at base 2221. The first mutation changes amino acid 68 of PreM from arginine to lysine and the second changes amino acid 454 of E from threonine to asparagine. Translation of pD2ME transcripts is expected to begin at codon 103 of Den-2 (base 330), which is the signal sequence for PreM, and proceed through amino acid 462 of E. Following amino acid 462 are the seven nonDen-2 amino acids (methionine, glutamic acid, leucine, serine, arginine, proline, and leucine) and a termination codon.

To determine if the constructs properly express the Den-2 E gene, 293 cells were transiently transfected with pkCMVintPolyli and pD2ME and analyzed by immune precipitation. The autoradiogram of the SDS-PAGE immune-precipitates shows that Den-2 polyclonal HIAF precipitates a protein of the expected size of truncated E from media of cells transfected with pD2ME (Figure 1A, lane 2), but not from the media of cells transfected with pkCMVintPolyli (lane 1). Cell lysates of pD2ME transfected cells were immune-precipitated with polyclonal Den-2 HIAF (Figure 1B, lane 2) and two monoclonal antibody mouse ascitic fluids that recognize conformational epitopes on the dengue virus envelope, 4G2 (lane 4) and 3H5 (lane 6). The ability of the monoclonal

Table 1 IFA of transfected DNA plasmids\*

	Den-2 HIAF	4G2	3H5
pkCMVintPolyli	-	-	-
pVR1012	-	-	-
pD2ME	++	++	++
p1012D2ME	+++	+++	+++

\*IFA of transfected cells. DNAs transfected and the antisera used are listed. The slides were rated on a fluorescence scale of - (negative) to 4+ (four positive)

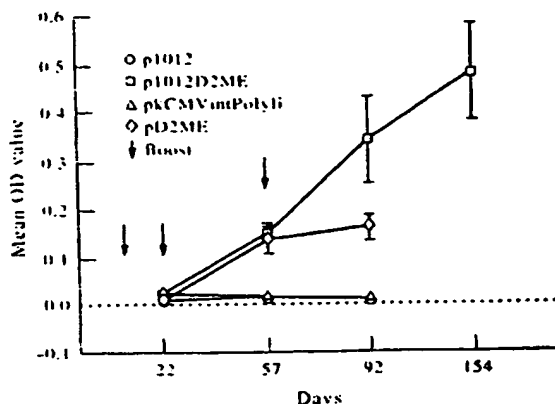


Figure 2 Anti-Den-2 antibodies by ELISA. Mean ELISA O.D. values vs days post-DNA inoculation is shown. Each point represents the mean O.D. plus or minus the standard error at the indicated days post-inoculation

antibodies to recognize the truncated E protein suggests that the protein is folding normally. None of the antibody preparations recognized a protein of the size of truncated E from cell lysates of cells transfected with pkCMVintPolyli (Figure 1B, lanes 1, 3, and 5).

IFA was performed on cells transfected with pkCMVintPolyli, pVR1012, pD2ME, and p1012D2ME. Mouse polyclonal HIAF and the two monoclonal antibodies, 4G2 and 3H5, reacted with cells transfected with pD2ME and p1012D2ME (Table 1). The intensity of fluorescence was consistently greater for cells transfected with p1012D2ME, which suggests that this construct produces a greater amount of truncated E than pD2ME. No fluorescence was detected with cells transfected with pkCMVintPolyli or pVR1012.

#### Mouse immunization and detection of anti-Den antibodies

Groups of ten, 3-week-old Balb/c mice were intradermally inoculated with 200 µg of pkCMVintPolyli, pVR1012, pD2ME, or p1012D2ME. The mice received three boosts and blood samples were tested for Den antibodies as described in the Materials and Methods section. The dilutions used in the ELISA were 1:25 for sera obtained after the first and second boosts and 1:100 for sera obtained after the third boost. No Den antibodies were detected following the initial dose of either vaccine candidate; however, after the second DNA boost, nine of ten mice that received pD2ME and ten of ten mice that received p1012D2ME had detectable Den-2 antibodies in the ELISA (Figure 2). After the third DNA boost, all mice that received pD2ME or

Table 2 Neutralizing antibody titers in mice immunized with dna plasmids\*

Animal No.	PRNT <sub>50</sub>	
	p1012D2ME	pD2ME
1	320	40
2	80	40
3	160	20
4	80	80
5	320	40
6	320	80
7	10	80
8	20	40
9	320	20
10	320	40

\*Results expressed as the reciprocal dilution of the PRNT<sub>50</sub> titer

p1012D2ME were positive by ELISA for Den-2 antibodies. The p1012D2ME inoculated mice were again assayed for Den-2 antibodies on day 154. The ELISA O.D. values continued to increase. The mice that received pkCMVintPolyli or pVR1012 produced no Den-2 antibodies.

Sera from mice obtained 35 days after the third boost with p1012D2ME or pD2ME were tested for Den-2 virus neutralization activity by PRNT. As controls, sera from pVR1012 and pkCMVintPolyli inoculated mice were also tested. The Den-2 PRNT<sub>50</sub> in the sera of mice inoculated with p1012D2ME ranged from 1:10 to 1:320 (median 240) (Table 2). The neutralizing titers in the sera of mice inoculated with pD2ME ranged from 1:20 to 1:80 (median 40). Sera from pVR1012 and pkCMVintPolyli inoculated mice contained no virus neutralizing activity.

#### Den-2 challenge of vaccinated mice

Groups of 6-week-old mice that had received one boost of pVR1012 or p1012D2ME were challenged with a lethal dose of Den-2 New Guinea C virus. No significant protection was afforded the mice inoculated with p1012D2ME.

#### DISCUSSION

This is the first report of DNA immunization of mice using Den genes. Our vaccine constructs, pD2ME and p1012D2ME, contained the preM gene and 92% of the E gene from Den-2 virus. The E gene was chosen for inclusion in the DNA vaccine construct because monoclonal antibody mapping and recombinant protein immunization studies with flaviviruses have shown that it contains the major epitopes responsible for eliciting neutralizing antibodies<sup>2</sup>. This gene was truncated at the carboxy terminus to remove the hydrophobic membrane anchor segment in order to maximize the secretion of protein. The preM gene also was included because the preM protein is thought to prevent low pH-induced irreversible conformational changes in the E protein as it is processed through the acidic compartments of the Golgi complex prior to secretion<sup>2,4</sup>.

The pD2ME and p1012D2ME constructs were derived from the plasmids pkCMVintPolyli and pVR1012 respectively. The pVR1012 plasmid differs from pkCMVintPolyli by deletion of the SV-40 origin of replication and the replacement of the SV-40 polyadenylation signal

with a bovine growth hormone polyadenylation site. The p1012D2ME construct showed a higher level of *in vitro* expression by IFA compared to the pD2ME construct. *In vivo*, the p1012D2ME construct was also more immunogenic than the pD2ME construct. The mean O.D. value and the median neutralizing antibody titer were twofold and sixfold higher, respectively, for sera from mice immunized with the p1012D2ME construct compared to sera from the pD2ME immunized animals. These data indicate that deleting the SV-40 origin of replication and SV-40 polyadenylation site from the plasmid backbone enhanced the expression and immunogenicity of the cloned Den-2 PreM and 92% E genes; however, the contribution of each of these plasmid changes to the enhanced expression cannot be determined from our data. The mean O.D. values for sera from mice immunized with p1012D2ME have not leveled off by day 92, but continued to increase linearly out to day 154. Possibly, this may be due to continued expression of antigen which has been seen in other nucleic acid inoculation systems<sup>15</sup>.

The E protein expressed from pD2ME and p1012D2ME reacted with monoclonal antibodies 3H5 and 4G2. The 3H5 monoclonal antibody is directed against a Den-2 serotype-specific epitope and the 4G2 against a flavivirus group-specific epitope on the E protein. Both epitopes are conformation dependent. Immunoreactivity with these antibodies indicates that the same epitopes on the expressed E are similar to the epitopes on the native E of Den-2. The additional seven nonDen amino acids and the conserved point mutations in the E gene on pD2ME and p1012D2ME appeared to have no significant effect on the antigenicity of the construct.

A total of four DNA injections were administered to the mice following boosting intervals similar to those used for other DNA vaccines<sup>25-28</sup>. The need for multiple boosts is not clear, since a single injection has been shown to elicit long term immune responses<sup>29</sup>. Currently other doses and boosting regimens are being explored.

Challenge experiments conducted in parallel using the DNA constructs failed to protect mice against lethal Den-2 virus challenge. In this challenge model, mice are immunized beginning at 3 weeks of age and challenged at 6 weeks of age by giving intracranial injections of lethal amounts of the virus. Mice older than 6 weeks of age are less susceptible to Den virus infection<sup>30</sup>. This model has proven to be suitable for evaluating the protective efficacy of live virus and recombinant vaccine candidates<sup>30-31</sup> capable of eliciting anti-Den immune responses by 6 weeks of age. Antibody responses generated by pD2ME and p1012D2ME were absent at 6 weeks and were not detectable until the mice were 11-weeks-old. This provides a clear explanation for the failure of the DNA constructs to protect against lethal Den-2 challenge. In a suitable model, the high Den-2 neutralizing antibody titers obtained by the DNA vaccines should confer protection since immunization studies with live and inactivated Den-2 virus have conferred protection in the mouse model at even lower neutralizing antibody levels<sup>34</sup>. Also, studies with other flaviviruses using vaccinia recombinant preM-E constructs have elicited similar levels of neutralizing antibody and conferred solid protection in mice against homologous live virus challenge<sup>35,36,37</sup>. Presently, studies are being conducted to evaluate the protective

efficacy of p1012D2ME Den antibodies in a murine passive transfer model.

The results presented in this report demonstrate the ability of p1012D2ME and pD2ME to elicit neutralizing antibody responses in mice and are considered Den-2 vaccine candidates, which warrant further study. We are presently testing similar constructs, expressing genes from the other dengue virus serotypes, in the hopes of developing a tetravalent dengue virus vaccine.

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## REFERENCES

- 1 Russel, P. and Nisalak, A. Dengue virus identification by the plaque reduction neutralization test. *J. Immunol.* 1967, **99**, 291-296
- 2 Chambers, T.J., Hahn, C.S., Galler, R. and Rice, C.M. Flavivirus genome organization, expression, and replication. *A. Rev. Microbiol.* 1990, **44**, 649-688
- 3 Halstead, S.B. Selective primary health care: strategies for control of disease in the developing world. XI. Dengue. *Rev. Infect. Dis.* 1984, **6**, 251-264
- 4 Halstead, S.B. Pathogenesis of dengue: challenges to molecular biology. *Science* 1988, **239**, 476-481
- 5 Schlesinger, R.W. *Dengue Viruses*. Springer-Verlag Wien, New York, 1977
- 6 Eckels, K.H., Dubois, D.R. and Summers, P.L. et al. Immunization of monkeys with baculovirus-dengue type-4 recombinants containing envelope and nonstructural proteins: evidence of priming and partial protection. *Am. J. Trop. Med. Hyg.* 1994, **50**, 472-478
- 7 Deubel, V., Kinney, R. and Esposito, J. et al. Dengue-2 virus envelope protein expressed by a recombinant vaccinia virus fails to protect monkeys against dengue. *J. Gen. Virol.* 1988, **69**, 1921-1929
- 8 Edelman, R., Tacket, C.O. and Wasserman, S.S. et al. A live attenuated dengue-1 vaccine candidate (45AZ5) passaged in primary dog kidney cell culture is attenuated and immunogenic for humans. *J. Infect. Dis.* 1994, **170**, 1448-1455
- 9 McKee, K.T.Jr, Bancroft, W.H., Eckels, K.H., Redfield, R.R., Summers, P.L. and Russell, P.K. Lack of attenuation of a candidate dengue 1 vaccine (45AZ5) in human volunteers. *Am. J. Trop. Med. Hyg.* 1987, **36**, 435-442
- 10 Bhamarapavati, N., Yoksan, S., Chayanitayothin, T., Angsubhakorn, S. and Bunyaratvej, A. Immunization with a live attenuated dengue-2-virus candidate vaccine (16681-PDK 53): clinical, immunological and biological responses in adult volunteers. *Bull. Wild Hlth Organ.* 1987, **65**, 189-195
- 11 Sedegah, M., Hedstrom, R., Hobart, P. and Hoffman, S. Protection against malaria by immunization with plasmid DNA encoding circumsporozoite protein. *Proc. Natl Acad. Sci. USA* 1994, **91**, 9866-9870
- 12 Wang, B., Boyer, J. and Srikanthan, V. et al. DNA inoculation induces neutralizing immune responses against human immunodeficiency virus type 1 in mice and non-human primates. *DNA Cell Biol.* 1993, **12**, 799-805
- 13 Ulmer, J., Donnelly, J. and Parker, S. et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 1993, **259**, 1745-1749

- 14 Major, M., Vitvitski, L. and Mink, M. *et al.* DNA-based immunization with chimeric vector for the induction of immune responses against hepatitis C virus nucleocapsid. *J. Virol.* 1995, 69, 5798-5805
- 15 Rhodes, G., Dwarki, V. and Abai, A. *et al.* Injection of expression vectors containing viral genes induces cellular, humoral, and protective immunity. *Vaccines* 1993, 93, 137-141
- 16 Sambrook, J., Fritsch, E. and Maniatis, T. *Molecular Cloning a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989
- 17 Megret, F., Hugnot, J.P. and Falconar, A. *et al.* Use of recombinant fusion proteins and monoclonal antibodies to define linear and discontinuous antigenic sites on the dengue virus envelope glycoprotein. *Virology* 1992, 187, 480-491
- 18 Hencchal, E., Gentry, M., McCown, J. and Brandt, W. Dengue virus specific and flavivirus group determinants identified with monoclonal antibodies by indirect immunofluorescence. *Am. J. Trop. Med. Hyg.* 1982, 31, 830-836
- 19 Raz, E., Carson, D.A. and Parker, S.E. *et al.* Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc. Natl Acad. Sci. USA* 1994, 91, 9519-9523
- 20 Cole, G.A., Charles, J. and Wisseman, L. Pathogenesis of type 1 dengue virus infection in suckling, weanling and adult mice. *Am. J. Epidemiol.* 1969, 89, 669-680
- 21 Ansari, M., Shope, R. and Malik, S. Evaluation of vero cell lysate antigen for the ELISA of flaviviruses. *J. Clin. Lab.* 1993, 7, 230-237
- 22 Eckels, K., Brandt, W., Harrison, V., McCown, J. and Russell, P. Isolation of temperature sensitive dengue 2 virus under conditions suitable for vaccine development. *Infect. Immun.* 1976, 14, 1221-1227
- 23 Gruenberg, A., Woo, W.S., Biedrzycka, A. and Wright, P.J. Partial nucleotide sequence and deduced amino acid sequence of the structural proteins of dengue virus type 2, new guinea C and PUO-218 strains. *J. Gen. Virol.* 1988, 69, 1391-1398
- 24 Allison, S.L., Stadler, K., Mandl, C.W., Kunz, C. and Heinz, F.X. Synthesis and secretion of recombinant tick-borne encephalitis virus protein E in soluble and particulate form. *J. Virol.* 1995, 69, 5816-5820
- 25 Ulmer, J.B., Deck, R.R., DeWitt, C.M., Friedman, A., Donnelly, J.J. and Liu, M.A. Protective immunity by intramuscular injection of low doses of influenza virus DNA vaccines. *Vaccine* 1994, 12, 1541-1544
- 26 Coney, L., Wang, B. and Ugen, K.E. *et al.* Facilitated DNA inoculation induces anti-HIV-1 immunity *in vivo*. *Vaccine* 1994, 12, 1545-1550
- 27 Davis, H.L., Michel, M.-L., Mancini, M., Schleef, M. and Whalen, R.G. Direct gene transfer in skeletal muscle: plasmid DNA-based immunization against the hepatitis B virus surface antigen. *Vaccine* 1994, 12, 1503-1509
- 28 Webster, R.G., Fynan, E.F., Santoro, J.C. and Robinson H. Protection of ferrets against influenza challenge with a DNA vaccine to the haemagglutinin. *Vaccine* 1994, 12, 1495-1500
- 29 Yankaukas, M.A., Morrow, J.E. and Parker, S.E. *et al.* Long-term anti-nucleoprotein cellular and humoral immunity is induced by intramuscular injection of plasmid dna containing NP gene. *DNA Cell Biol.* 1993, 12, 771-776
- 30 Bray, M., Zhao, B.T., Markoff, L., Eckels, K.H., Charock, R.M. and Lai, C.J. Mice immunized with recombinant vaccinia virus expressing dengue 4 virus structural proteins with or without nonstructural protein NS1 are protected against fatal dengue virus encephalitis. *J. Virol.* 1989, 63, 2853-2856
- 31 Delenda, C., Frenkiel, M.P. and Deubel, V. Protective efficacy in mice of a secreted form of recombinant dengue-2 virus envelope protein produced in baculovirus infected insect cells. *Arch. Virol.* 1994, 139, 197-207
- 32 Feighny, R., Burrous, J. and Putnak, R. Dengue type-2 virus envelope protein made using recombinant baculovirus protects mice against virus challenge. *Am. J. Trop. Med. Hyg.* 1994, 50, 322-328
- 33 Fonseca, B.A., Pincus, S., Shope, R.E., Paoletti, E. and Mason, P.W. Recombinant vaccinia viruses co-expressing dengue-1 glycoproteins prM and E induce neutralizing antibodies in mice (published erratum appears in *Vaccine* 1994, 12, 480). *Vaccine* 1994, 12, 279-285
- 34 Delenda, C., Frenkiel, M. and Deubel, V. Protective efficacy in mice of a secreted form of recombinant dengue-2 virus envelope protein produced in baculovirus infected insect cells. *Arch. Virol.* 1994, 139, 197-207
- 35 Konishi, E., Pincus, S., Paoletti, E., Shope, R. and Mason, P. Avipox virus-vectored Japanese Encephalitis virus vaccines: use as vaccine candidates in combination with purified subunit immunogens. *Vaccine* 1994, 12, 633-638
- 36 Pincus, S., Mason, P. and Konishi, E. *et al.* Recombinant vaccinia virus producing the prM and E proteins of yellow fever virus protects mice from lethal yellow fever encephalitis. *Virology* 1992, 187, 290-297
- 37 Venugopal, K., Jiang, W. and Gould, E. Immunity to St. Louis encephalitis virus by sequential immunization with recombinant vaccinia and baculovirus derived PrM/E proteins. *Vaccine* 1995, 13, 1000-1005

# Immunity to St. Louis encephalitis virus by sequential immunization with recombinant vaccinia and baculovirus derived PrM/E proteins

K. Venugopal\*†, W.R. Jiang\* and E.A. Gould\*

*St. Louis encephalitis (SLE) is an important mosquito-borne disease of great public health concern in parts of the United States, South America and Canada. Protective immunogens of flaviviruses produced in different expression systems have been shown to be effective against virulent virus infection in laboratory animal models. Here we show that the pre-membrane and envelope (PrM/E) of SLE virus expressed in insect and mammalian cell systems using baculovirus and vaccinia virus, respectively, are processed correctly and showed similar antigenic characteristics as the authentic proteins. Immunization with the recombinant proteins individually or in combination resulted in neutralizing and protective immune responses. A schedule consisting of initial immunization with recombinant vaccinia virus followed by a secondary boost with recombinant baculovirus protein resulted in higher levels of neutralizing and protective immune responses. The advantages of the use of such a combined approach as a general immunization strategy are discussed.*

**Keywords:** Recombinant vaccine; St. Louis encephalitis virus; PrM/E protein; baculovirus/vaccinia virus

St. Louis encephalitis (SLE) is an important mosquito-borne virus disease affecting humans in the United States, South America and Canada. Since its first recognition, nearly 60 years ago, it has been responsible for at least 10 000 cases and numerous deaths. In addition to sporadic cases of SLE occurring each year, there have also been large suburban and urban epidemics involving thousands of cases that appear to occur in 7–10 year cycles. It is believed that infected migrating birds spread the virus across America and into Canada<sup>1</sup>. However, in general, SLE virus does not produce large-scale widespread epidemics and therefore vaccine development has not been a priority in the past.

An inactivated mouse brain-derived vaccine prepared shortly after the second world war using the Webster strain of SLE virus was shown to be relatively effective in protecting humans against this disease<sup>2</sup>. With recent advances in biotechnology, it is now possible to avoid the potential problems associated with mouse brain tissues by utilizing recombinant DNA technology to

design potential vaccines against a number of flavivirus diseases<sup>3</sup>. However, strategies are required to ensure effective humoral and T-cell immune responses against the virus. Recombinant live virus vaccines offer some advantages over either inactivated virus or expressed protein vaccines, since their multiplication results in sustained expression of the gene product *in vivo* and presentation to the immune system, in a manner more closely simulating natural infection.

Vaccinia virus (VV) represents an attractive vector for flavivirus vaccine development<sup>4,5</sup>. However, one potential drawback to the general use of recombinant vaccinia is that pre-existing immunity to VV might limit its replication and therefore interfere with the immune response to the recombinant antigen<sup>6,7</sup>. Flavivirus proteins can be produced in large quantities using recombinant baculovirus expression systems<sup>8</sup> and they provide an alternative immunogen which is not dependent on replication in the host. Immunization with such proteins has been shown to be safe and effective against many flaviviruses<sup>9</sup>. In an attempt to combine the advantages of each of these vaccine preparations in inducing protection against SLE virus, we conducted a study to determine if a sequential immunization approach consisting of priming with recombinant VV expressing prM/E proteins and boosting with a similar protein derived from baculoviruses will stimulate a more effective protective response to SLE virus.

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SLE prM/E

1000 Vaccine 1995 Volume 13 Number 11

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## MATERIALS AND METHODS

### Cells and viruses

CV-1 TK<sup>-</sup> and CV1 TK<sup>-</sup> cells (American type culture collection) were grown in Dulbeccos modified Eagles medium with 5% foetal calf serum (FCS) at 37°C in 5% CO<sub>2</sub>. *Spodoptera frugiperda* (Sf9) cells were cultured at 28°C in TC 100 medium (Gibco) containing 5% FCS and 100 U ml<sup>-1</sup> kanamycin. The MSI-7 strain of SLE virus<sup>10</sup>, the WR strain of wild-type VV and the genetically modified strain of baculovirus<sup>11</sup> were used.

### Cloning of the PrM/E gene and construction of recombinant transfer vectors

SLE virus RNA was extracted from infected suckling mouse brain preparations using standard methods<sup>12</sup>. First strand cDNA was synthesised using antisense primer 5'ACGGGATCCCTAGCTAATTGCACATC 3' (complementary to the SLE virus sequence nucleotide position 2441–2460) using Superscript reverse transcriptase (BRL). The polymerase chain reaction (PCR) was used (30 cycles: 1.5 min at 94°C, 1 min at 50°C and 2 min at 72°C) to amplify the genome segment from the signal sequence of the PrM protein to the C-terminus of the E glycoprotein using the antisense primer described above and a sense primer 5'CTAGGATCCATAACATGGGGCC-AGCAAGAAG 3' (derived from the SLE virus sequence nucleotide positions 372–404). The PCR product was purified from agarose gels and cloned into pGEM-T vector (Promega). The *Bam*HI fragment from the cDNA clone was ligated to the *Bam*HI site of pAcYMI baculovirus transfer vector<sup>13</sup> and *Bgl*II site of VV transfer vector pSC11<sup>14</sup>. The recombinant vectors were checked for the correct transcriptional orientation by restriction mapping and sequencing across the *Bam*HI junctions using oligonucleotide primers derived from the vector sequences.

### Generation of recombinant baculovirus and VV

Transfection and selection of recombinant baculoviruses were performed as previously described<sup>13</sup> using wild-type baculovirus DNA linearised with *Bsu*361<sup>11</sup>. Plaque-purified recombinant baculovirus stocks with infectivity titres of 10<sup>7</sup>–10<sup>8</sup> p.f.u. ml<sup>-1</sup> were used in all subsequent experiments. Recombinant VV expressing SLE virus proteins were selected using methods previously described<sup>8</sup>. Recombinant virus was plaque purified 3 times in selective growth medium before generation of a high titre virus stock (10<sup>7</sup>–10<sup>8</sup> p.f.u. ml<sup>-1</sup>) which was used in all subsequent experiments. A monoclonal antibody (MAb 813) which is broadly cross-reactive against the E protein of flaviviruses<sup>15</sup> was used in indirect immunofluorescence (IF) tests for the initial screening and selection of recombinant viruses.

### SDS-PAGE and immunoblotting of recombinant protein

Recombinant baculovirus protein for electrophoresis was prepared by infecting Sf9 cells with baculovirus (m.o.i. 10) and the cells, harvested 48 h post-infection (p.i.), were washed once in cold PBS (pH 7.2) and lysed in TNN buffer (10 mM Tris-HCl pH 7.4, 0.5% Nonidet P-40 (NP-40) and 100 mM NaCl). Similar methods were

followed 12 h post-infection, for preparing infected CV-1 cell lysates for the recombinant VV protein. The proteins were resolved on 10% SDS-polyacrylamide gels<sup>16</sup> and were transferred onto nitrocellulose membranes using the semi-dry technique. The membranes were blocked for 30 min in PBS supplemented with 5% non-fat milk and 0.05% Tween 20 and incubated for 2 h at room temperature (RT) in a 1:500 dilution of rabbit anti-SLE hyperimmune serum. After repeated washing in PBS-Tween 20, the membranes were incubated for 1 h at RT with 1:3000 dilution of peroxidase-conjugated anti-rabbit IgG (BRL). The unbound antibodies were removed by repeated washings and the recombinant proteins were revealed by reacting with 0.05% DAB (Sigma) solution containing 1% H<sub>2</sub>O<sub>2</sub>.

### Radiolabelling and analysis of the proteins (RIP)

To prepare radiolabelled baculovirus proteins, confluent monolayers of Sf9 cells in 35 mm dishes were infected with recombinant or wild-type baculovirus (10 m.o.i.) for 22 h at 28°C. The cells were washed and incubated for 1 h with methionine-free medium and then labelled with (<sup>35</sup>S)methionine (100 Ci μl<sup>-1</sup>) for 1 h. Tissue culture supernatant medium was collected and cells were recovered and washed in cold PBS, and solubilized in 100 μl of RIPA buffer (0.02 M Tris HCl pH 7.4, 0.15 M NaCl, 0.01 M EDTA, 1% NP-40 and 0.01 M phenyl-methylsulfonyl fluoride). The post-nuclear supernatant was collected and stored at –20°C. A similar protocol was followed for obtaining the radiolabelled proteins of VV and SLE virus using CV-1 cells at infection times of 12 and 30 h, respectively. In experiments designed to study the glycosylation patterns of the proteins, tunicamycin (10 μg ml<sup>-1</sup>) was added to the growth medium at 18 h p.i. of infected Sf9 cells, at 2 h p.i. of CV-1 cells infected with VV and 24 h p.i. of CV-1 cells infected with SLE virus. Aliquots (100 μl) of the lysates were immunoprecipitated as follows. Samples were incubated for 16 h at 4°C with 2 μl of a rabbit anti-SLE hyperimmune serum. Immune complexes were precipitated using *Staphylococcus* protein A-Sepharose (Sigma) and washed free of unbound proteins and label. The immunoprecipitated proteins were resolved by 10% SDS-PAGE and visualized by autoradiography.

### Antigenic analysis of recombinant proteins by IF tests

The antigenic properties of recombinant proteins were compared in an IF test using a panel of MAbs and methods described previously<sup>15</sup> on Sf9 and CV-1 cells infected with recombinant baculovirus and VV, respectively.

### Animal protection experiments

Mouse protection experiments were performed as previously described<sup>8</sup>. Immunizing antigen from recombinant baculovirus was obtained from infected Sf9 cells (10<sup>7</sup> cells ml<sup>-1</sup>) lysed by repeated freezing and thawing. After centrifugation at 2000 revs min<sup>-1</sup> to remove the cell debris, 0.1 ml was administered subcutaneously with an equal amount of aluminium hydroxide adjuvant. Immunization with VV was done by intraperitoneal

administration of  $10^7$  p.f.u. of wild-type or recombinant VV prepared by lysis of infected CV-1 cells by repeated freeze-thaw cycles and sonication. Groups ( $n=10$ ) of 3-week-old outbred TO mice (Tuck and Sons, UK) were immunized as per the following regimen.

Group 1 Wild-type VV at 0 day+wild-type baculovirus at 14 day

Group 2 Recombinant baculovirus at 0 day+recombinant baculovirus at 14 day

Group 3 Recombinant VV at 0 day+recombinant VV at 14 day

Group 4 Recombinant VV at 0 day+recombinant baculovirus at 14 day.

Two weeks after the second immunization, all the animals were inoculated intracerebrally with 0.01 ml of  $10^{-4}$  dilution of infected suckling mouse brain ( $10^8$  p.f.u. ml $^{-1}$ ) of MSI-7 strain of SLE virus. Following SLE virus infection, mice were observed daily for development of symptoms.

#### Antibody response to vaccination

The antibody response to the recombinant proteins in the vaccinated mice was determined by ELISA<sup>1</sup> using 96-well plates (Dynatech Immulon) coated with sonicated, glycerol-tartrate gradient purified, SLE virus antigen. Before testing, non-specific binding sites were blocked with PBS containing 2% non-fat milk and 0.025% Tween-20. Pooled sera from randomly selected mice, from each group, were diluted twofold starting from 1:100 and 100  $\mu$ l of each dilution was added to the wells and incubated for 1 h at 37°C. After thorough washing, 100  $\mu$ l of affinity-purified horseradish peroxidase-conjugated sheep anti-mouse IgG (BRL) was added at a dilution of 1:500 and incubated for 1 h at 37°C. The plates were washed 5 times and the bound peroxidase activity was detected colorimetrically using ABTS<sup>2</sup>, 2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid), as the substrate, on an ELISA reader at 405 nm.

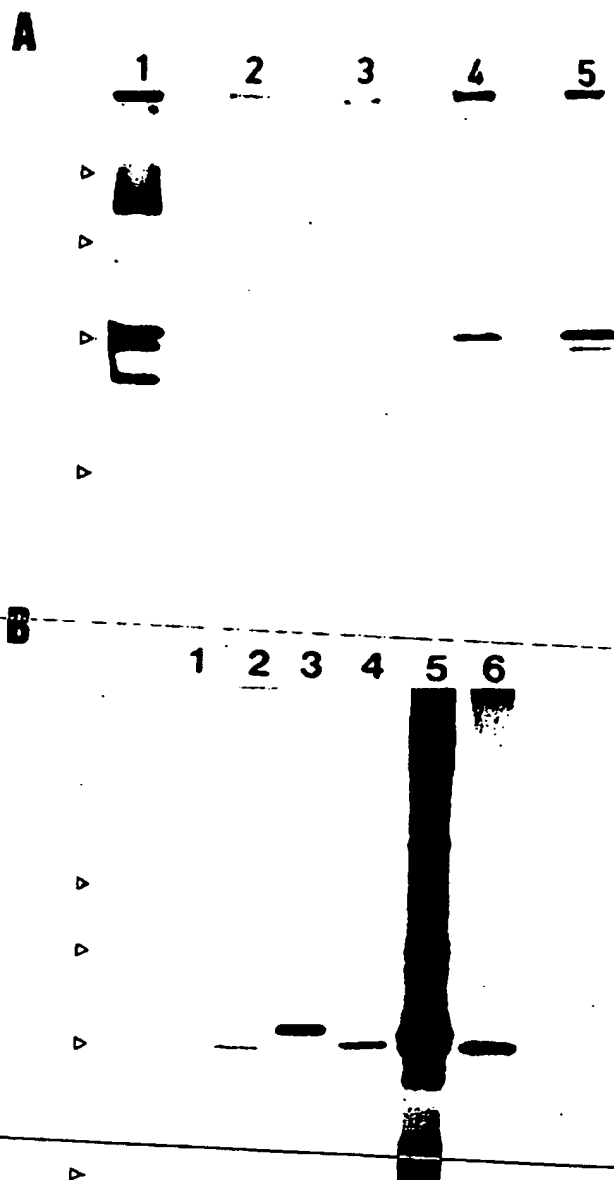
#### Virus neutralization tests (NT)

These were performed on the pooled sera from each group of mice essentially as described previously<sup>1</sup> with a  $10^{-4}$  dilution of an infectious tissue culture stock of SLE virus. The titre was expressed as the reciprocal of the highest dilution of antibody that caused a 50% reduction of plaque numbers relative to the virus control.

## RESULTS

#### Analysis of the expressed recombinant proteins

Screening by IF tests of S/9 and CV-1 cells infected with the recombinant baculovirus and VV, respectively, using a panel of MAbs, gave identical results<sup>18</sup> to SLE virus infected PS cells (not shown). Immunoblotting of the infected S/9 and CV-1 cell lysates using the SLE virus hyperimmune antiserum showed that the expressed recombinant E protein migrated to the same position as SLE virus E protein. Although the recombinant virus constructs contained both PrM and E coding sequences, no PrM/M proteins were detectable. This was probably because the antiserum did not contain high titre antibodies against M protein. Cell lysates infected with SLE



**Figure 1** (A) Radioimmunoprecipitation of SLE virus proteins with polyclonal antiserum. Lane 1—PS cell lysates infected with SLE virus; lane 2—S/ cell lysate infected with WT baculovirus; lane 3—CV1 cell lysate infected with WT vaccinia virus; lane 4—S/ cell lysate infected with recombinant baculovirus; lane 5—CV1 cell lysate infected with recombinant vaccinia virus. (B) Effect of tunicamycin on the mobilities of radiolabelled SLE virus proteins. Labelled PS cells infected with SLE virus in the absence (lane 1) and presence (lane 2) of tunicamycin; labelled CV1 cells infected with recombinant vaccinia virus in the absence (lane 3) and presence (lane 4) of tunicamycin; labelled S/ cell lysate infected with recombinant baculovirus in the absence (lane 5) and presence (lane 6) of tunicamycin. Arrows represent protein molecular weight markers from the top 97, 66, 45 and 31 kDa

virus also failed to demonstrate the presence of M protein when tested with the same antiserum. Analysis of the proteins by RIP with SLE virus-specific antiserum confirmed the similarity of the recombinant protein and SLE virus E protein. Both proteins migrated as a doublet on PAGE gel with a molecular mass of 51–54 kDa inferring differences in the glycosylation status of

**Table 1** Immune response of mice to recombinant SLE virus

Mouse group	ELISA titre <sup>a</sup>	Neutralisation titre <sup>b</sup>
1	<20	<10
2	200	60
3	800	120
4	1600	320

<sup>a</sup>Reciprocal of the highest dilution of O.D. value more than twice the negative control value. <sup>b</sup>Reciprocal of the highest dilution that caused a 50% reduction in the number of plaques

the two bands (*Figure 1A*). In order to confirm whether or not the faster migrating protein represented the unglycosylated form, radiolabelled proteins synthesised in the presence of tunicamycin were analysed using RIP with antiserum against SLE virus. Tunicamycin treatment resulted in disappearance of the 54 kDa band (*Figure 1B*), demonstrating that the upper band was glycosylated.

#### Analysis of the antibody response to recombinant SLE E antigen

Pre-challenge pooled sera of randomly selected mice from different groups, immunized as described earlier, were initially tested for their ability to immunoprecipitate radiolabelled SLE virus proteins. The results of these studies demonstrated that the recombinant proteins had induced antibody responses in mice (not shown). When the serum samples were tested in an ELISA to compare the immune response evoked due to different vaccination schedules, animals in group 4 showed the highest titres (*Table 1*). The serum samples from the different groups also showed differences in neutralization titres.

#### Protective immune responses against virulent SLE virus infection

The ability of the recombinant viruses, used under different immunization schedules, to protect mice against virulent SLE virus infection was tested by intracerebral challenge of immunized mice with SLE virus as described above. All animals in group 1, which were not immunized with SLE virus proteins, died within 7 days, confirming the virulent nature of the SLE virus challenge. Significant protective immune responses against SLE virus were observed in groups of mice immunized with either recombinant baculovirus (60% survival) or recombinant VV expressing SLE virus PrM/E proteins (80%). However, the animals in group 4 which had been immunized first with recombinant VV and then with recombinant baculovirus expressing SLE virus proteins, all survived infection with a lethal challenge dose of SLE virus (*Figure 2*). The experiments were repeated and gave identical results.

## DISCUSSION

Recent advances in biotechnology have made it possible to develop recombinant antigens as potential protective immunogens against different virus diseases. Recombinant virus constructs expressing the structural proteins PrM and E have been shown to elicit protective immune

responses against the mosquito-borne flaviviruses Japanese encephalitis, yellow fever and dengue type 4 virus<sup>3</sup>. Several investigators have used either baculovirus<sup>10</sup> or VV<sup>4,5</sup> to derive the recombinant flavivirus antigens for immunization. In order to harness the advantages of both expression systems, we examined the effects of immunizing mice with recombinant PrM/E proteins of SLE virus using VV in the first instance followed by recombinant baculovirus derived protein as a secondary booster antigen for inducing protective responses against virulent SLE virus infection.

Both types of recombinant virus expressed a protein which migrated at the same position as SLE virus E protein and both reacted with SLE virus-specific polyclonal antiserum in immunoblot and RIP tests. The recombinant virus PrM/E protein also showed an identical reactivity pattern with SLE virus protein when tested with a panel of E-specific MAb. The sequence of the structural proteins of SLE virus shows that cellular signalases may be responsible for proteolytic cleavage of the PrM and E proteins<sup>10</sup>. The identical size and antigenic reactivity of the recombinant E protein also suggests proper processing and folding of these proteins in mammalian and insect cells. Recent studies have shown that prM protein was important in protecting the E protein during its processing in the highly acidic pH of the post-Golgi vesicles<sup>20</sup> and recombinant constructs expressing prM/E proteins of mosquito-borne viruses such as JE and YF produced extracellular particles capable of inducing protective immune responses<sup>4,5</sup>. The SLE virus prM protein produced by the recombinant viruses may also have a similar function.

Differences in the glycosylation patterns between SLE virus strains have been reported, although they do not appear to correlate with antigenicity or biological behaviour<sup>21</sup>. The MSI-7 strain of SLE virus used in the present study, has two potential N-linked glycosylation sites on the E protein and SLE virions in a population may contain both glycosylated and non-glycosylated E proteins. The recombinant E protein behaved like the authentic protein and migrated as two bands differing in glycosylation status, as seen in tunicamycin inhibition experiments (*Figure 2*).

Effective virus vaccines can stimulate antibodies that neutralize virus infectivity and bind to cell-free virus to aggregate it. They can also stimulate cytolytic effector cells to eliminate virally infected cells during infection. This requirement for a broad effector response may not be easy to achieve with subunit vaccines derived from one type of recombinant virus, since antigens presented in different ways may vary in efficiency to induce individual components of the immune response. The group of mice that received only recombinant baculovirus derived PrM/E protein showed a moderate humoral antibody response (*Table 1*). Mice which received two doses of recombinant VV also showed good antibody responses, indicating that it may be possible to enhance the immune response by repeated administration of live recombinant viruses. However, the data from the antibody studies and protection from intracerebral challenge with SLE virus show that secondary boosting with antigen from a different source may be more advantageous. Studies with *Plasmodium yoelii* have shown that administration of a second dose of the same recombinant virus failed to enhance the immunity probably due



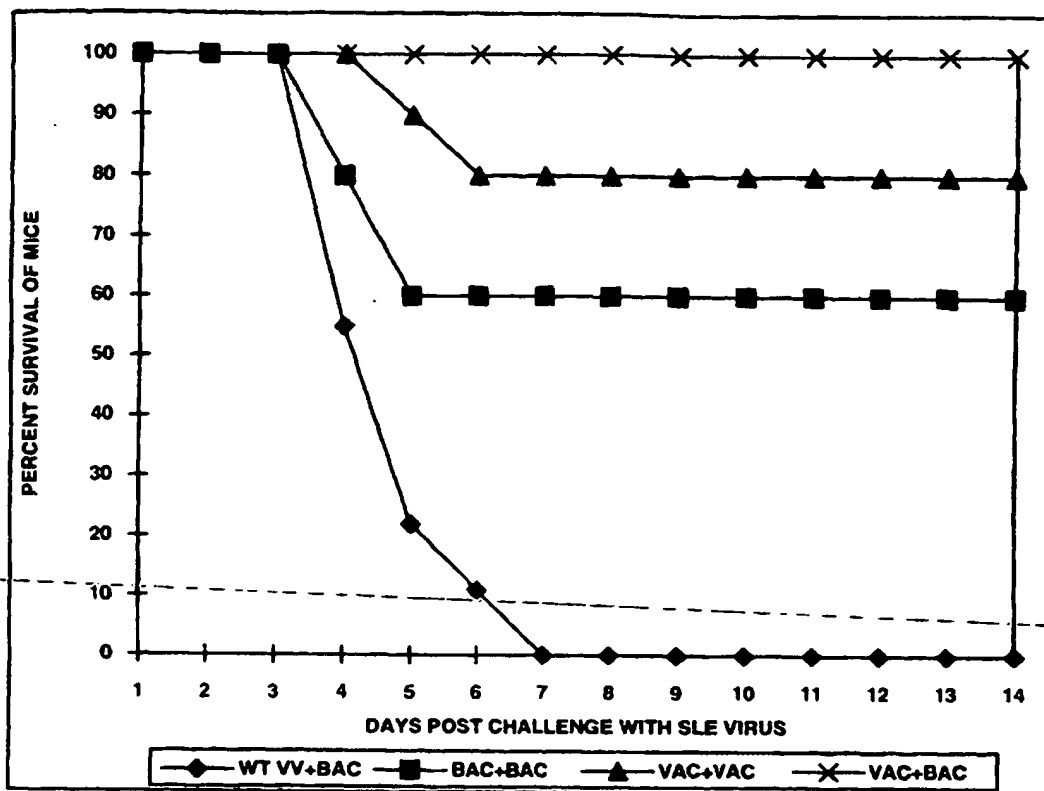


Figure 2 Survival data for groups ( $n=10$ ) of mice inoculated with WT or recombinant viruses and then challenged intracerebrally with a lethal dose of SLE virus

to the vigorous primary response that rapidly neutralizes the second dose of the virus<sup>22</sup>.

Our results show that the SLE virus PrM/E proteins, expressed by recombinant virus have the potential for use as a subunit vaccine and this supports the conclusions<sup>3-5,23</sup> that flavivirus vaccines in general could be constructed using this principle. Among the different immunization regimens that were compared, the most successful was seen when mice were immunized with recombinant vaccinia virus followed by a secondary boost with recombinant baculovirus protein. This finding was consistent with the higher antibody response observed in the group given the combined immunization schedule. Although issues such as the durability of the immune response, the interval for further boosting and the use of more potent adjuvants require additional investigation, our data show that recombinant virus constructs of PrM and E proteins can function as efficient immunogens against SLE virus and that the use of a combined vaccination approach should be considered as a general immunization strategy for flaviviruses.

## REFERENCES

- 1 Chamberlain, R.W. History of St. Louis encephalitis. In: *St. Louis Encephalitis* (Ed. Monath, T.P.). American Public Health Association, Washington, D.C., 1988, pp. 3-61
- 2 Sabin, A.B., Duffy, C.E., Warren, J., Ward, R., Peck, J.L. and Ruchmann, I. The St. Louis and Japanese B types of epidemic encephalitis. *J. Am. Med. Assoc.* 1943, 122, 477-481

- 3 Venugopal, K. and Gould, E.A. Towards a new generation of flavivirus vaccines. *Vaccine* 1994, 12, 966-975
- 4 Konishi, E., Pincus, S., Paoletti, E., Shope, R.E., Burrage, T. and Mason, P.W. Mice immunized with a subviral particle containing the Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. *Virology* 1992, 188, 714-719
- 5 Pincus, S., Mason, P.W., Konishi, E. et al. Recombinant vaccinia virus producing the prM and E proteins of yellow fever virus protects mice from lethal yellow fever encephalitis. *Virology* 1992, 187, 290-296
- 6 El-Ad, B., Roth, Y., Winder, A. et al. The persistence of neutralizing antibodies after revaccination against smallpox. *J. Infect. Dis.* 1990, 161, 446-448
- 7 Rooney, J.F., Wohlenberg, C., Cremer, K.J., Moss, B. and Notkins, A.L. Immunization with a vaccinia virus recombinant expressing herpes simplex virus type glycoprotein D: long term protection and effect of revaccination. *J. Virol.* 1988, 62, 1530-1534
- 8 Shiu, S.Y.W., Reid, H.W. and Gould, E.A. Louping ill virus envelope protein expressed by a recombinant baculovirus and vaccinia virus fails to stimulate protective immunity. *Virus Res.* 1992, 26, 213-222
- 9 Zhang, Y.M., Hayes, E.P., McCarty, T.C. et al. Immunization of mice with dengue structural proteins and nonstructural protein NS1 expressed by baculovirus recombinant induces resistance to dengue virus encephalitis. *J. Virol.* 1988, 62, 3027-3031
- 10 Trent, D.W., Kinney, R.M., Johnson, B.J. et al. Partial nucleotide sequence of St. Louis encephalitis virus RNA: structural proteins, NS1, ns2a and ns2b. *Virology* 1987, 156, 293-304
- 11 Kitts, P.A., Ayres, M.D. and Possee, R.D. Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. *Nucl. Acids Res.* 1991, 18, 5667-5672
- 12 Chomczynski, P. and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem.* 1987, 162, 156-159

- 13 Matsuura, Y., Possee, R.D., Overton, H.A. and Bishop, D.H.L. Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. *J. Gen. Virol.* 1987, **68**, 1233-1250
- 14 Chakrabarti, S., Brechling, K. and Moss, B. Vaccinia virus expression vector: coexpression of  $\beta$ -galactosidase provides visual screening of recombinant virus plaques. *Molec. Cell. Biol.* 1985, **5**, 3403-3409
- 15 Gould, E.A., Buckley, A., Cammack, N. *et al.* Examination of the immunological relationships between flaviviruses using yellow fever virus monoclonal antibodies. *J. Gen. Virol.* 1985, **66**, 1369-1382
- 16 Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* 1970, **227**, 680-685
- 17 Jiang, W.R., Bonnett, T.P., Venugopal, K. and Gould, E.A. A single chain antibody fragment expressed in bacteria neutralizes tick-borne flaviviruses. *Virology* 1994, **200**, 21-26
- 18 Venugopal, K., Buckley, A., Reid, H.W. and Gould, E.A. Nucleotide sequence of the envelope glycoprotein of Negishi virus shows very close homology to louping ill virus. *Virology* 1992, **190**, 515-521
- 19 Deubel, V., Bordier, M., Megret, F., Gentry, M.K., Schlesinger, J.J. and Girard, M. Processing, secretion and immunoreactivity of carboxy-terminally truncated dengue-2 envelope proteins expressed in insect cells by recombinant baculoviruses. *Virology* 1991, **180**, 442-447
- 20 Guirakhoo, R., Bolin, R.A. and Roehrig, J.T. The Murray Valley encephalitis virus PrM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of the E glycoprotein. *Virology* 1992, **191**, 921-931
- 21 Vordam, V., Mathews, J.H., Barrett, A.D.T., Roehrig, J.T. and Trent, O.W. Molecular and biological characterization of a non-glycosylated isolate of St. Louis encephalitis virus. *J. Gen. Virol.* 1993, **74**, 2653-2660
- 22 Li, S., Rodrigues, M., Rodriguez, D. *et al.* Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces CD8<sup>+</sup> T-cell-mediated protective immunity against malaria. *Proc. Natl Acad. Sci. USA* 1993, **90**, 5214-5218
- 23 Venugopal, K., Shiu, S.Y.W. and Gould, E.A. Recombinant vaccinia virus expressing prM and E glycoproteins of louping ill virus: induction of partial homologous and heterologous protection in mice. *Res. Vet. Sci.* 1994, **57**, 188-193



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**United States Patent** [19]**Dubensky, Jr. et al.**[11] **Patent Number:** **5,814,482**[45] **Date of Patent:** **Sep. 29, 1998****[54] EUKARYOTIC LAYERED VECTOR INITIATION SYSTEMS**

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[21] **Appl. No.:** **739,158**[22] **Filed:** **Oct. 30, 1996****Related U.S. Application Data**

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[51] **Int. Cl.<sup>6</sup>** ..... **C12P 21/00; C12N 15/86; C07H 21/04**

[52] **U.S. Cl.** ..... **435/69.3; 435/320.1; 536/23.1; 536/24.1**

[58] **Field of Search** ..... **435/69.3, 320.1; 536/23.1, 24.1**

**[56] References Cited****U.S. PATENT DOCUMENTS**

5,091,309 2/1992 Schlesinger et al. .... 435/69.1  
5,185,440 2/1993 Davis et al. .... 536/23.72  
5,217,879 6/1993 Huang et al. .... 435/69.1

**FOREIGN PATENT DOCUMENTS**

WO 92/10578 6/1992 WIPO .  
WO 94/17813 8/1994 WIPO .  
WO 95/07994 3/1995 WIPO .  
WO 95/17525 6/1995 WIPO .  
WO 95/27044 10/1995 WIPO .  
WO 95/27069 10/1995 WIPO .  
WO 95/31565 11/1995 WIPO .  
WO 95/32733 12/1995 WIPO .

**OTHER PUBLICATIONS**

Boyer and Haenni, "Infectious Transcripts and cDNA Clones of RNA Viruses," *Virology* 198: 415-426, 1994.

Dubensky, Jr. et al., "Sindbis Virus DNA-Based Expression Vectors: Utility for In Vitro and In Vivo Gene Transfer," *Journal of Virology* 70(1): 508-519, 1996.

Herweijer et al., "A Plasmid-Based Self-Amplifying Sindbis Virus Vector," *Human Gene Therapy* 6: 1161-1167, 1995.

Kuhn et al., "Infectious RNA Transcripts from Ross River Virus cDNA Clones and the Construction and Characterization of Defined Chimeras with Sindbis Virus," *Virology* 182: 430-444, 1991.

Liljeström and Garoff, "A New Generation of Animal Cell Expression Vectors Based on the Semliki Forest Virus Replicon," *BioTechnology* 9: 1356-1361, 1991.

Rice et al., "Production of Infectious RNA Transcripts from Sindbis Virus cDNA Clones: Mapping of Lethal Mutations, Rescue of a Temperature-Sensitive Marker, and In Vitro Mutagenesis To Generate Defined Mutants," *Journal of Virology* 61(12): 3809-3819, 1987.

Xiong et al., "Sindbis Virus: An Efficient, Broad Host Range Vector for Gene Expression Animal Cells," *Science* 243: 1188-1191, 1989.

Grakoui et al., "A cis-Acting Mutation in the Sindbis Virus Junction Region Which Affects Subgenomic RNA Synthesis," *Journal of Virology* 63(12): 5216-5227, 1989.

Hertz and Huang, "Utilization of Heterologous Alphavirus Junction Sequences as Promoters by Sindbis Virus," *Journal of Virology* 66(2): 857-864, 1992.

Levis et al., "Promoter for Sindbis Virus RNA-Dependent Subgenomic RNA Transcription," *Journal of Virology* 64(4): 1726-1733, 1990.

Polo et al., "Alphavirus mediated delivery of ribozyme therapeutics," *J. Cell. Biochem., Suppl.*, 19A: 288, abstract no. A6-413, 1995.

Schlesinger, S., "Alphaviruses—vectors for the expression of heterologous genes," *Trends in Biotechnology* 11: 18-22, 1993.

Baumann and Schendel, "Interleukin-11 Regulates the Hepatic Expression of the Same Plasma Protein Genes as Interleukin-6," *Journal of Biological Chemistry* 266: 20424-20427, 1991.

Davis et al., "In Vitro Synthesis of Infectious Venezuelan Equine Encephalitis Virus RNA from a cDNA Clone: Analysis of a Viable Deletion Mutant," *Virology* 171: 189-204, 1989.

Huang et al., "RNA viruses as Gene Expression Vectors," *Virus Genes* 3(1): 85-91, 1989.

Oker-Blom and Summers, "Expression of Sindbis Virus 26S cDNA in *Spodoptera frugiperda* (Sf9) Cells, Using a Baculovirus Expression Vector," *Journal of Virology* 63(3): 1256-1264, 1989.

Racaniello and Baltimore, "Cloned Poliovirus Complementary DNA Is Infectious in Mammalian Cells," *Science* 214: 916-919, 1981.

Rice et al., "Expression of Sindbis Virus Structural Proteins via Recombinant Vaccinia Virus: Synthesis, Processing, and Incorporation into Mature Sindbis Virions," *Journal of Virology* 56(1): 227-239, 1985.

Semler et al., "Production of infectious poliovirus from cloned cDNA is dramatically increased by SV40 transcription and replication signals," *Nucleic Acids Research* 12(12): 5123-5141, 1984.

(List continued on next page.)

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[57]

**ABSTRACT**

The present invention provides compositions and methods for utilizing recombinant alphavirus vectors. Also disclosed are compositions and methods for making and utilizing eukaryotic layered vector initiation systems.

**25 Claims, 30 Drawing Sheets**

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## OTHER PUBLICATIONS

- Wen and Schlesinger, "Regulated expression of Sindbis and vesicular stomatitis virus glycoproteins in *Saccharomyces cerevisiae*," *Proc. Natl. Acad. Sci. USA* 83: 3639-3643, 1986.
- Berglund et al., "Semliki Forest Virus Expression System: Production of Conditionally Infectious Recombinant Particles," *BioTechnology* 11: 916-920, 1993.
- Bredenbeek and Rice, "Animal RNA virus expression systems," *Seminars in Virology* 3: 297-310, 1992.
- Bredenbeek et al., "Sindbis Virus Expression Vectors: Packaging of RNA Replicons by Using Defective Helper RNAs," *Journal of Virology* 67(11):6439-6446, 1993.
- Davis et al., "Protection Against Influenza in Mice By Vaccination With a Venezuelan Equine Encephalitis Virus Vector Expressing the HA Protein," *J. Cell. biochem. Suppl.* 19A: 310. Abstract No. J2-308, 1995.
- Geigenmüller-Gnirke et al., "Complementation between Sindbis viral RNAs produces infectious particles with a bipartite genome," *Proc. Natl. Acad. Sci. USA* 88: 3253-3257, 1991.
- Johanning et al., "A sindbis virus mRNA polynucleotide vector achieves prolonged and high level heterologous gene expression in vivo," *Nucleic Acids Research* 23(9): 1495-1501, 1995.
- Levis et al., "Deletion Mapping of sindbis Virus DI RNAs Derived from cDNAs Defines the Sequences Essential for Replication and Packaging," *Cell* 44: 137-145, 1986.
- Levis et al., "Engineered defective interfering RNAs of sindbis virus express bacterial chloramphenicol acetyltransferase in avian cells," *Proc. Natl. Acad. Sci. USA* 84: 4811-4815, 1987.
- Liljeström, P., "Alphavirus expression systems," *Current Opinion in Biotechnology* 5: 495-500, 1994.
- Olivo et al., "A Cell Line That Expresses a Reporter Gene in Response to Infection by Sindbis Virus: A Prototype for Detection of Positive Strand RNA Viruses," *Virology* 198: 381-384, 1994.
- Owen and Kuhn, "Identification of a Region in the Sindbis Virus Nucleocapsid Protein That Is Involved in Specificity of RNA Encapsidation," *Journal of Virology* 70(5): 2757-2763, 1996.
- Raju and Huang, "Analysis of Sindbis Virus Promoter Recognition In Vivo. Using Novel Vectors with Two Sub-genomic mRNA Promoters," *Journal of Virology* 65(6): 2501-2510, 1991.
- Rolls et al., "Expression of Additional Genes in a Vector Derived from a Minimal RNA Virus," *Virology* 218:406-411, 1996.
- Rolls et al., "Novel Infectious Particles Generated by Expression of the Vesicular Stomatitis Virus Glycoprotein from a Self-Replicating RNA," *Cell* 79: 497-506, 1994.
- Sarver and Stollar, "Sindbis Virus-Induced Cytopathic Effect in Clones of *Aedes albopictus* (Singh) Cells," *Virology* 80: 390-400, 1977.
- Strauss and Strauss, "The Alphaviruses: Gene Expression, Replication, and Evolution," *Microbiological Reviews* 58(3): 491-562, 1994.
- Strauss et al., "Complete Nucleotide Sequence of the Genomic RNA of Sindbis Virus," *Virology* 133: 92-110, 1984.
- Watson et al., "A Mutant CHO-K1 Strain with Resistance to Pseudomonas Exotoxin A and Alphaviruses Fails To Cleave sindbis Virus Glycoprotein PE2," *Journal of Virology* 65(5): 2332-2339, 1991.
- Weiss and Schlesinger, "Recombination between Sindbis Virus RNAs," *Journal of Virology* 65(8): 4017-4025, 1991.
- Weiss et al., "Evidence for Specificity in the Encapsidation of Sindbis Virus RNAs," *Journal of Virology* 63: 5310-5318, 1989.
- Weiss et al., "Interactions between Sindbis virus RNAs and a 68 amino acid derivative of the viral capsid protein further defines the capsid binding site," *Nucleic Acids Research* 22(5): 780-786, 1994.
- Zhou et al., "Generation of cytotoxic and humoral immune responses by nonreplicative recombinant Semliki Forest virus," *Proc. Natl. Acad. Sci. USA* 92: 3009-3013, 1995.
- Zhou et al., "Self-replicating Semliki Forest virus RNA as recombinant vaccine," *Vaccine* 12(6): 1510-1514, 1994.
- International Search Report, PCT Patent Application No. PCT/US94/10469, May 4, 1995.
- European Search Report, EP Patent Application No. 95115460.8, Nov. 15, 1996.

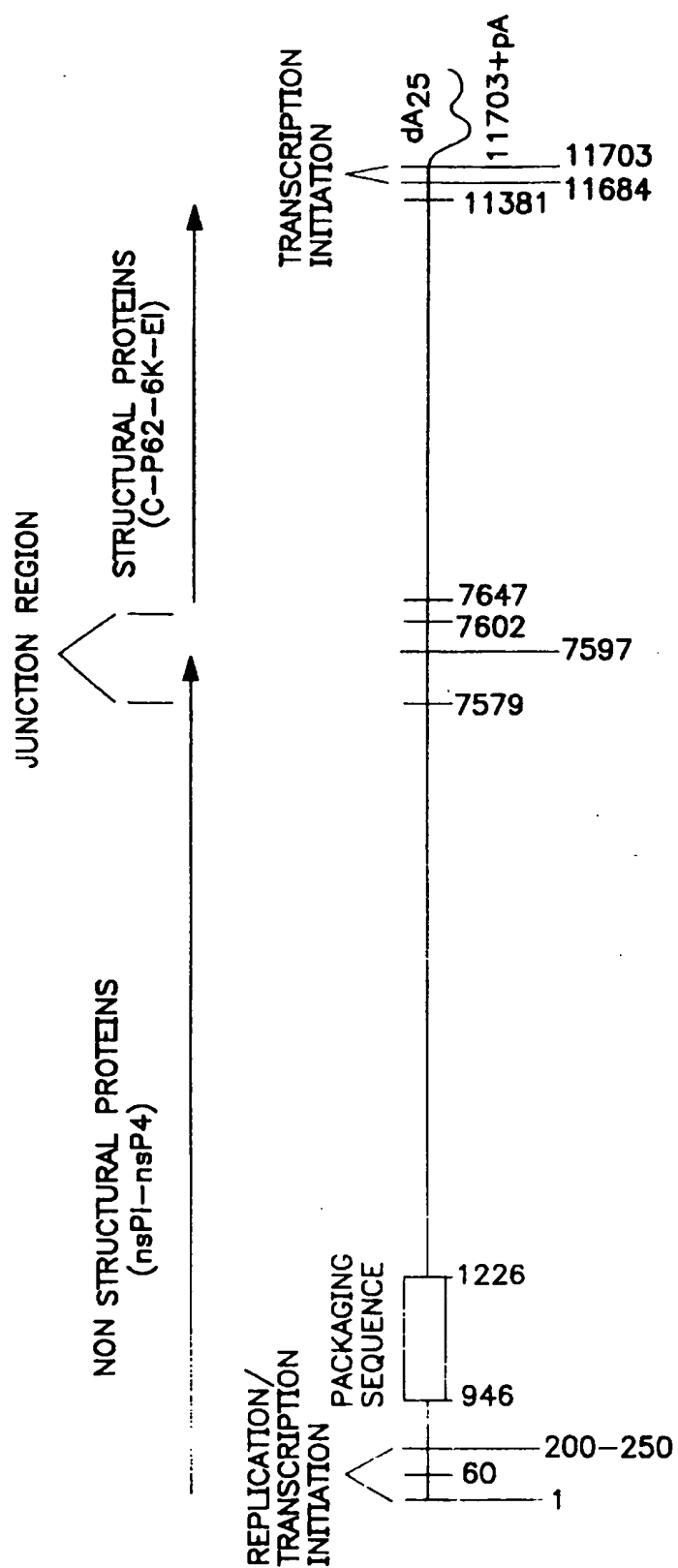


FIG. 1

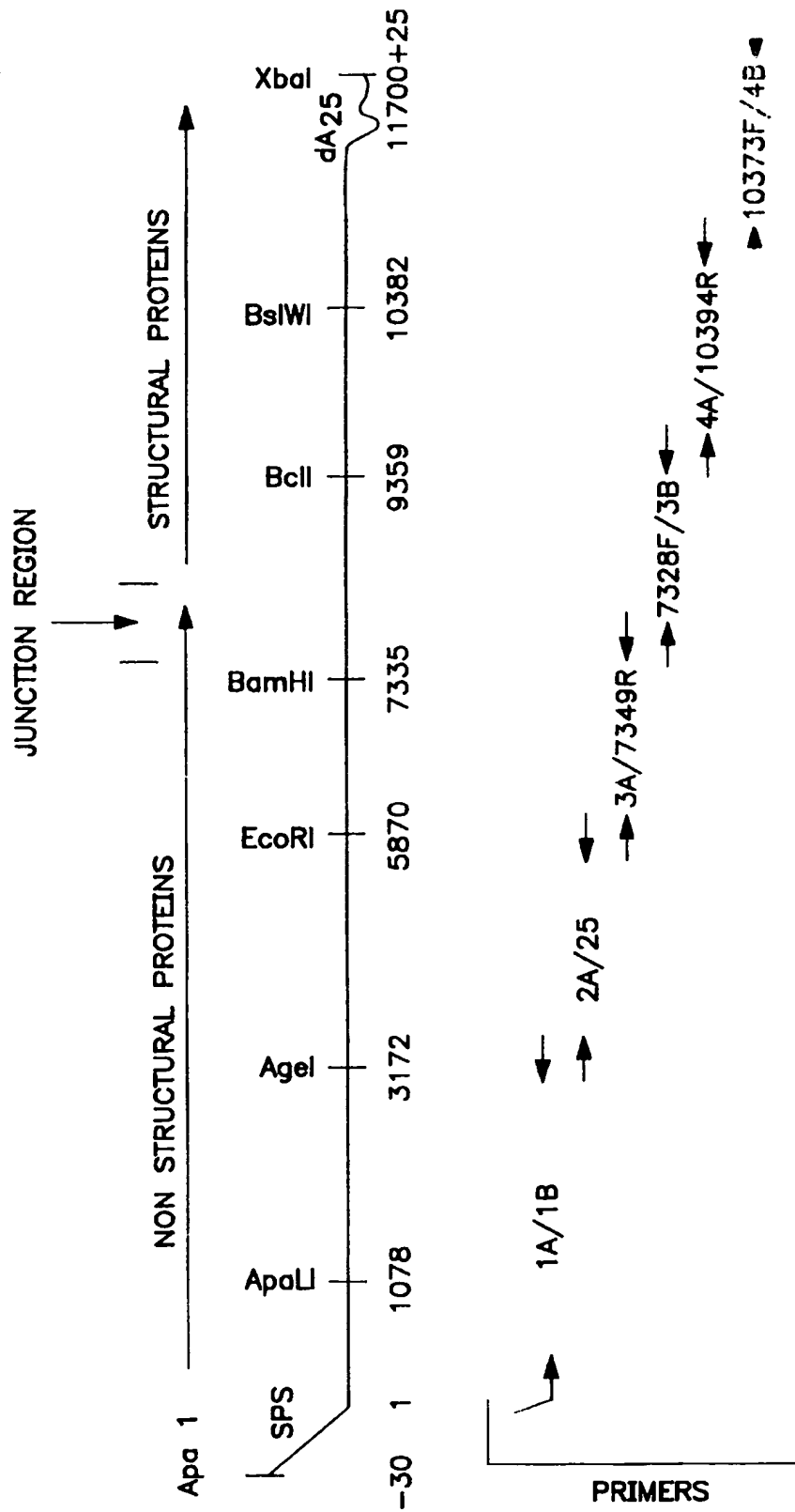


FIG. 2

FIG. 3A

ATTGACGGCG  
TGGAGAAGCC  
AAAAAAGCTT  
ATGCCAGAGC  
CGACGATCTT  
ATTGTGCTCG  
AACTGGCGGA  
TCCGGACCGT  
TTACCTGCAA  
GAACTATCTA  
CCACCCAGTT  
CCGACGAGAA  
GTAGGACAGG  
ATTTCTCCGT  
TTCCATCGGT  
TGAGTTGCCA  
CCGTGGGATA  
CAGTAAAGG  
ATCAGATGAC  
TTGGGCTCAA  
AAAAATTACCT  
ATGATCTTGA  
TGTGGCGT  
GCGTAAAGT  
TGCCCATGTC  
TGCCTCAGGT  
AGGAAGCCAG  
TCGAGGCAGC  
CATTAGTTGA  
TCGGACAGTA  
CGACCCCGCT  
CGGTCCGACC  
GCAAACTATA  
AGGTTACAAA  
GCGTTAAGAA  
ATCATGAGCT  
CAATAGGAGT  
TAGTACACAC  
AGTAGTAAAC  
CCCGCAATTT  
ATTTTCGCAT  
GGACATAGGC  
CCCCATGCGT  
AAAAAGCTGC  
AACTGGATCG  
CATGCGTGCC  
TCATCAGGCT  
CATGTTCTCG  
AGTCTTGAA  
AAAAATTGTCG  
AGGATCGACA  
GTTCCACTTG  
AGGCTACGTA  
CGCGGTTACA  
AGAACGGGTA  
TGGTCTAATG  
CCAGCGAATT  
TCTGCCGATC  
TAACGAGAAA  
TCGCACTAAG  
CCCAGCCTCT  
GCTGAGGCAG  
CTCGGAGGAA  
AGCGGAGAAG  
CGCAGAAGTT  
AACCCCGCGC  
TATCGTTGTC  
AGCAGATCAG  
ATACGACGCT  
ACTGAGTGAG  
CCACATTGCC  
GCGAGAGCTT  
GGCAGAGCTT  
GGAAGAAGCC  
AGCTCTGGAG  
GATAGGCACA  
TATTGAATCA  
GTAGCGTAG  
GAGGTAGTAG  
CTGGCCAGTA  
AGCGCACCGG  
AGTCCAGAAG  
AAGATTACAA  
CCGGAATGCTG  
GAAATTCCG  
ATGAAAGGCG  
GCTATGGCAG  
GGCGTAACA  
ATAATGAGGA  
CTTTATCCAG  
AATGGAAAGC  
GTGAAAGAAA  
CACAAATAGCG  
TCGTTCCCTG  
GCCACGGATA  
GTCAATTACG  
ATAGCACAA  
ATGCTGGGTA  
AAAGTACATT  
TTTAGCGCTT  
AAATTGAAC  
TTAGTCATGG  
CTCCGAGAAG  
GTCTGCGAAG  
GGTACGTA  
TCGCCAAACT  
GTTAAGATCA  
AAAGTACTGA  
AGCGCCACGT  
ATGATGAGT  
ATGCAATGGC  
GCAGAAACAG  
TCAGGCTG  
GGACTGAAGA  
CCGGGGTCGG  
TATTGAATCA  
AACAGCCGAC  
ACCCCCAGAG  
CACAGCAGGT  
AACAATCGA  
CTCGTAGAAT  
ACCCGGACCG  
ACCAAGAACT  
ACAAGAACT  
AAACACCATC  
TCAATGAGGA  
TGCGGACCC  
GTTCTGACCC  
TCGGACTTTG  
AGAAGGAGTT  
AACACAGAGC  
AGTCGTACAC  
TCACCATCAG  
AGGGCTCTT  
TGTGCAGTA  
TATCACCTGA  
GTAGGACTAA  
GGTTCAGCAA  
CTAGAGAAGC  
CGTTTTATCG  
TCCCCATGTC  
TGGCATTGCA  
AGGCCAAGGC  
CACTTCCACC  
TGGAGGGGCT  
GGATAATACC  
CTGTGCTGAA  
TAACACACTC  
TGCCAGCAGG  
TAGTGTACAA  
CCGCCAAGAA  
AGTACGTGTT  
TCCTCTCGGG  
CCCGACCTGC  
GCAAGTCCGC  
AACAGCCGAC  
GACCAATCGA  
GCTTTCGAG  
ATTAGTGGCA  
CCAGGCGGAC  
TCAAGCAAAT  
GAATGCCAAA  
CGGAAGATCA  
AGGTGCCGTA  
CGAAAGAGAG  
TACAGAAGAG  
TGACGTGGAC  
AGAACTGACC  
GGTCCCGTAC  
TATTATCAAG  
CAATCGCACT  
TCCGTTTGT  
GACTCCAAAT  
GCTGAGGTT  
GTTTTCCGAG  
CATGATGAAA  
GCATGAGAAG  
GCCTGCTATC  
CTGTGATATC  
TGCTGATAC  
GAGCACAAAG  
GAAGCCCGG  
CAGCTTGCAG  
TTGCCGCTGT  
TCCCGGGATC  
GCTATGCAAA  
CATCCCGGCC  
CGATCCGACAA  
CAGGAACACC  
ATGGGCTAAG  
CAAGCTTACG  
CCCACCTGGA  
GTCCGTATGG  
ACCAAGAAG  
TGC TTTTGG  
ATTAGTGGCA  
CCAGGCGGAC  
TCAAGCAAAT  
GAATGCCAAA  
CGGAAGATCA  
AGGTGCCGTA  
CGAAAGAGAG  
TACAGAAGAG  
TGACGTGGAC  
AGAACTGACC  
GGTCCCGTAC  
TATTATCAAG  
ACCATCACAA  
GTGCATATGC  
GACCAATGCTA  
CACCAGTATG  
AACACCATGC  
GAGCGCAAGG  
TATGGCTGCT  
ACGCAGACCT  
ACGACCTCTT  
ACGAAATAAC  
GATGCTCAGG  
GACAAAGGCA  
ATCGGAGCAG  
GACCGTATGA  
CTCGCACCCAG  
GGAAGGTACG  
CCATGGCCAG  
TTTGTGAACC  
GGGAGGTACG  
AAGAAGCGTT  
AACCTCCCT  
AAGGTCGAAA  
TCAACTGTCA

FIG. 3B

GCGCACGAGA	TCTTGTTTACC	AGCGSAAAGA	AAGAAAAATTG	TCGGGAAAATTT	GAGGCCGACG	2340
TGCTAAGACT	GAGGGGTATG	CAGATTACGT	CGAAGACAGT	AGATTTCGGTT	ATGCTCAACG	2400
GATGCCACAA	AGCCGTAGAA	GTGCTGTACG	TTGACGAAGC	GTTTCGGTGC	CACGCAGGAG	2460
CACACTATTG	CTTGATTGCT	ATCGTCAGGC	CCCGCAAGAA	GGTAGTACTA	TGCGGAGACC	2520
CCAATGCAATG	CGGATTCCTC	AACAATGATGC	AACATAAAGGT	ACATTTTCAAT	CACCCCTGA	2580
AAGACATAIG	CACCAAGACA	TTCTACAACT	ATATCTCCCG	ACATTTTCAAT	CAGCCAGTTA	2640
CAGCTATTGT	ATCGACACTG	CATTACGATG	GAAAGATGAA	GCGTTGCACA	CCGTGCAAGA	2700
AGAACAATTGA	AATCGATAIT	ACAGGGGCCA	CAAAGCCGAA	AACCACGGAAC	ATCATCTTGA	2760
CATGTTTTCCG	CGGGTGGGTT	AAGCAATTGC	AAATCGACTA	GCCCAGGGAT	GAAGTAATGA	2820
CAGCCGCGGC	CTCACAAAGG	CTAACCCAGAA	AAGGAGTGT	TCCCGGACAT	CAGAAAAGTCA	2880
ATGAANAACCC	ACTGTACGCG	ATCACATCAG	AGCATGTGAA	TGCCGTCGCG	ACCCGCACTG	2940
AGGACAGGCT	AGTGTGGAAA	ACCTTGCAGG	GCGACCCCATG	CGTGTGCTC	CTCACTAACA	3000
TACCTAAAGG	AAACITTCAG	GCTACTATAG	AGGACTGGGA	GATTAAGCAG	AAGGGAATAA	3060
TTGTCTGCAAT	AAACAGCCCC	ACTCCCCTGT	CCAAATCCGTT	AGCTGAACAC	ACCAACGTTT	3120
GTCTGGCGGAA	AGCATTTGGAA	CCGATTAATG	ATGCAAAACC	CAGCTGCAAG	ACCGTTTGCC	3180
AGTGGAGCGA	ACTGTTCCCA	CAGTTTGC	TGGACTTGAC	TATCGTACTT	ATTTACGCCT	3240
TAGACGTAAT	TTGCAITTAAG	TTTTTCGGCA	TGGACTTGAC	ACATTCGGCC	TTTTTCAAAAC	3300
ACTAACGTTAC	ACTAACGTTAC	CATCCC	ATTCAGCGAG	AGCCGGTAGCT	CATTGGGACA	3360
ACAGCCCCAGG	AACCCGCAAG	TATGGGTACG	ATCACGCCAT	TGCCGCGCGAA	CTCTCCCGTA	3420
GGATTTCCGGT	GTTCACGCTA	GCTGGGAAGG	GCACACAAC	TGATTTGCGAG	ACGGGGAGAA	3480
CCAGAGCTTGA	CTCTGCACAG	CATAACCTGG	TCCCGGTGAA	CCGCAATCTT	CCTCACGCCT	3540
TAGCCGCTTGA	GTACAAAGGAG	AAGCAACCCG	GCCCGGTCGA	AGCTCCCGT	AACCAAGTCA	3600
AGTACTTTGCT	AGTACTTTGCT	GTATCAGAGG	AAAAATTTGA	AGCTCCCGT	AAGAGAATCG	3660
CCCGATTGGC	CCCGATTGGC	ATAGCCGGTG	CAGATAAGAA	CTAACACCTG	GCTTCGGGT	3720
GGCAGGGTAC	GGCAGGGTAC	GACCTGGTGT	TCATCAACAT	TGGAACTTAA	TACAGAAACC	3780
GCAGTGC	GCAGTGC	GACCATGCGG	CGACCTTAA	AGCCCTTTTCG	CGTTAGGGCC	3840
CAACCCAGGA	CAACCCAGGA	GGCACCTCTG	TGGTGAAGTC	CTATGGCTAC	GCCGACCGCA	3900
CGTAGTCACC	CGTAGTCACC	GCTCTTGCCA	GAAAGTTTGT	CAGGGTGTCT	GCAGCGAGAC	3960
CTCAAGCAAT	CTCAAGCAAT	ACAGAAATGT	ACCTGATTTT	CCGACAACTA	GACAAACAGC	4020
ATTACCCCGC	ATTACCCCGC	CACCATCTGA	ATTGCGTGT	TTTCGTCCTG	TATGAGGGTA	4080
AGTTGGAGCC	AGTTGGAGCC	CGCCGCTCAT	ACCCGCTCAA	AAGGGAGAA	ATTGCTGACT	4140
AGCAGTTGTC	AGCAGTTGTC	AACGCAGCCA	ATCCGCTGGG	TAGACCCAGC	GAAGGAGTCT	4200
CTATAAACGT	CTATAAACGT	TGGCCGACCA	GTTTTACCGA	TTTCAGCCAGC	GAGACAGGCA	4260
GACTGTGTGC	GACTGTGTGC	CTAGGAAAGA	AAGTGATCCA	CGCGGTCGGC	CTGATTTCC	4320
AGAAGCAGAA	AGAAGCAGAA	GCCTTGAAAT	TGCTACAAA	CGCCTACCAT	GCAGTGGCAG	4380
TGAACATAAC	TGAACATAAC	ATCAAGTCTG	TCGCCATTCC	ACTGCTATCT	ACAGGCATTT	4440
AAAAGACCGC	AAAAGACCGC	CTTGAAGTAT	CACTTAACTG	CTTGACAACC	GCCTAGACAA	4500
GGACGTAACC	GGACGTAACC	ATCTATTGCC	TGGATAAGAA	GTGGAAGGAA	AGAAATCGACG	4560



**FIG. 3C**

CGGCACATCCCA	ACTTAAGGAG	TCGTGAACAG	AGCTGAAGGA	TGAAGATATG	GAGATCGACG	4620
ATGGAATCCAT	ATGGATCCAT	CCAGACAGTT	GCCTTGAAGGG	AAGAAAGGGA	TTTCAGTACTA	4680
ATTGATTCG	ATTGATTCG	TACTTCGAAG	GCACCAAAT	CCATCAAGCA	GCAAAAGACA	4740
AAAGGTCCTG	AAAGGTCCTG	TTCCCTAATG	ACCAGGAAAG	TAATGAACAA	CTGTGTGCC	4800
TGAGACCAATG	TGAGACCAATG	GAAGCAATCC	TTTGCATGTA	CCCGGTCGAC	CATAAACCGT	4860
GCCCAAAACG	GCCCAAAACG	TTGCCGTGCC	TTTGCATGTA	TGCCATGACG	CCAGAAAGGG	4920
TAGAAGCAAT	TAGAAGCAAT	AACGTCAAAG	AAGTTACAGT	ATGCTCTCTC	ACCCCTCTTC	4980
AAATTAAGAA	AAATTAAGAA	GTTCAGAAGG	TTTCAGTGCAC	GAAAGTAGTC	CTGTTLAATC	5040
CGCATTCGTT	CGCATTCGTT	CCCGCCCGTA	AGTACATAGA	AGTGCCAGAA	CAGCCTACCG	5100
ACAGGCCGAG	ACAGGCCGAG	GAGGCCCCCG	AAGTTGTAGC	GACACCGTCA	CCATCTACAG	5160
CTCGCTTGAT	CTCGCTTGAT	GTCACAGACA	CTCACTGGA	TATGGATGAC	AGTAGCGAAG	5220
TTTCGAGCTT	TTTCGAGCTT	AGCGGATCGG	ACCACTCTAT	TACTAGTATG	GACAGTTGGT	5280
TAGTTCACAT	TAGTTCACAT	GAGATAGTAG	ACCAGAGGCT	GGTGGTGGTG	GCCTGACGTT	5340
TGAGCCTTGCC	TGAGCCTTGCC	CCATATCCAC	CGCCAAAGGT	AAAGAAGATG	GCCCGGCTGG	5400
AAAAGAGCCC	AAAAGAGCCC	ACTCCACCCGG	CAAGCAATAG	CTCTGAGTCC	CTCCACCCTC	5460
GGATATCCATG	GGATATCCATG	TCCCCTCGAT	CAATTTTCGA	CGGAGAGACG	GCCCGGCCAGG	5520
ACCCCTTGCCA	ACCCCTTGCCA	ACAGGCCCCA	GAGATGTGCC	TATGCTTTTC	GGATCGTTTT	5580
GATTGTAGAG	GATTGTAGAG	CTGAGCCGCA	CGGCAACTGA	GTCCGAACCC	GTCCTGTTTT	5640
ACCGGGCGAA	ACCGGGCGAA	GTGAATCAA	TTATATCGTC	CCGATCAGCC	GTAATCTTTT	5700
GCAGAGACGT	GCAGAGACGT	AGACGCAGGA	GCAGGAGGAC	TGAAATACTGA	CTAACCCGGGG	5760
CATATTTTCG	CATATTTTCG	ACGGACACAG	GCCCCTGGGCA	CTTGGCAAAG	AAGTCCGTTT	5820
GCCTACAGAA	GCCTACAGAA	CCGACCTTGG	AGCGCAATGT	CCTGGAAAGA	ATTTCATGCC	5880
CACGTGCGAA	CACGTGCGAA	GAGGAACAAC	TCAAACCTAG	GTACCCAGATG	ATGCCCAACG	5940
AAGCTAGGTAC	AAGCTAGGTAC	CAGCTCTGTA	AGTAGAAACC	TCAGAAAGCC	ATAAACCACG	6000
GTCAGGACTAC	GTCAGGACTAC	GACTGTATA	ACTCTGCCAC	AGATCAGGCA	GAATGCTATA	6060
TCCGAAACCA	TCCGAAACCA	TTGTACTCCA	GTAGCGTACC	GCGGAACCTAC	TCCGATCCAC	6120
AGCTGCTGT	AGCTGCTGT	AACAACATC	TGCAATGAGAA	CTATCCGACA	GTAGCATCTT	6180
TGACCGAGTAC	TGACCGAGTAC	GATGCTTACT	TGGATATGGT	AGACGGGACA	GTCGCTGCC	6240
AACCTTCTGC	AACCTTCTGC	CCCGTTAAGC	TTAGAAAGTTA	CCCGAAACAA	CATGAGTATA	6300
TATCCCGCAGT	TATCCCGCAGT	CGGGTCCAT	CAGCGATGCA	GAACACGCTA	CAAAATGTGC	6360
AACATAAAGA	AACATAAAGA	AAATGCAACG	TCACGCAGAT	GCGTGAACATG	CCAACACTGG	6420
ATTCAATGTC	ATTCAATGTC	GAAATGCTTC	GAAAATATGC	ATGTAATGAC	GAGTATTGGG	6480
TCGGAAGCCA	TCGGAAGCCA	ATTAGGATTA	CCCACTGAGT	TGTCAACGCA	TATGTAGCTA	6540
CCCTAAGGCC	CCCTAAGGCC	GCCACACTAT	TTGCAAAGAC	GTATAAATTTG	GTCCCATTGC	6600
TATGGATAGA	TATGGATAGA	TTCGTCAATG	ACATGAAAG	AGACGTGAAA	GTTAACCCAG	6660
ACGACCGAAAG	ACGACCGAAAG	ATCATAGTGT	TATGAAAGT	ACAAGCCGCA	GTAACCCCTGG	6720
CTTATGCGGG	CTTATGCGGG	ATTTCGCGG	AAATAGTGG	TAGGCTTACG	GCCGTCTTGC	6780
TCACACGCTT	TCACACGCTT	TTTGACATGT	CGCGCGGAGGA	TTTTGTATGCA	ATCATAGCAG	6840

FIG. 3D

AACACTTCAA  
AAGACGACGC  
CACTACTCGA  
GTACTCGTTT  
ACACAGTTTT  
GATGTGCAGC  
TGCTGAGAG  
GTGAGAGACC  
CGTGCCGCGT  
ACGACGAGCA  
GAGTAGGTAT  
TTACACCTGT  
TCAGAGGGGA  
CTGACTAATA  
GCCCTTCCCG  
TGCTTGCCCG  
TAGTCATTGG  
AGAAGCAGGC  
AGAAGCAACC  
CCGACAGATT  
TGGAGGGAAA  
CAAAGCTCAA  
ACATGAGAAG  
ACACGGGAGC  
GAGGAGACAG  
GTGGCGCTGA  
AGACAAITAA  
CAATGTGTTT  
GCCGAACCTTC  
CCCTGCTCAA  
ACGACTTTAC  
CGTGCTTCAG  
GCATACAGAC  
ACCGCTACAT  
AGATTAGCAC  
AATGCCCTCC  
GTACACTGGC  
CCGTTACACG

GCAAGGGCGAC  
TATGGCGTTA  
CTTGATCGAG  
TAAATTCGGG  
GAAATGCTGT  
GTTCAATTGGC  
GTGCGCCACC  
ACCTTACTTC  
GGCGGATCCC  
AGACGAAGAC  
AACAGGCAC  
CCTACTGGCA  
AATAAAGCAT  
CTACAACACC  
GGCCCCCACT  
CAACGGGCTG  
ACAGGCAACT  
GCCCAAGCAA  
TGCAAAACCC  
GTTGACGCTC  
GGTAATGAAA  
ATTTACCAAG  
TGAGGCATTTC  
GGTGCAATAT  
CGGTGCTCCG  
TGAAGGAACA  
GACGACCCCG  
GCTCGGAAT  
CAGAGCCCTC  
TGCCATATTG  
CCTGACCCAGC  
CCCTGTTAAG  
TTCCGCCCCAG  
GTCGCTTAAG  
CTCAGGACCG  
AGGGGACAGC  
CCGCAAGATA  
TAAAAGAAAT

CCGGTACTGG  
ACCGGTCTGA  
TGCGCCTTTG  
GCGATGATGA  
ATCGCCAGCA  
GACGACAACA  
TGCTCAACA  
TGGCGCGGAT  
CTGAAAAGGC  
AGAAGACGCG  
TTAGCAGTGG  
TTGAGAACTT  
CTCTACGGTG  
ACCACTATGA  
GCCATGTGGA  
GCTTCTCAAA  
AGACCTCAAC  
CCACCGAAGC  
AAACCCGGAA  
AAGAACGAGG  
CCTCTGCACG  
TCGTGAGCAT  
ACCTACACCA  
AGTGAGGTA  
ATCATGGATA  
CGAACTGCC  
GAAGGGACAG  
GTGAGCTTCC  
GACATCCTTG  
CGGTGCGGAT  
CCCTACTTGG  
ATCGAGCAGG  
TTTGGATACG  
CAGGATCACA  
TGTAAGAGGC  
GTAACGGTTA  
AAACCAAAAT  
CCTTGCACAG

AGACGGATAT  
TGATCTTGG  
GAGAAATATC  
AATCCGGAAT  
GAGTACTAGA  
TCATACATGG  
TGGAGTTAA  
TTATCTTGCA  
TGTTAAGTT  
CTCTGCTAGA  
CCGTGACGAC  
TTGCCAGAG  
GTCTTAATA  
ATAGAGGATT  
GGCCGCGGAG  
TCCAGCACT  
CCCCACGTC  
CGAAGAAAC  
AGAGACAGCG  
ACGGAGATGT  
TGAAAGGAAC  
ACGACATGGA  
GTGAACACCC  
GATTTACCAT  
ACTCCGGTCG  
TTTCGGTCGT  
AAGAGTGGTC  
CATGCGACCG  
AAGAGAACGT  
CGTCTGGCAG  
GCACATGCTC  
TCTGGGACGA  
ACCAAGCGG  
CCGTTAAAGA  
TTAGCTACAA  
GCATAGTGAG  
TCGTGGGACG  
TGTACGACCG

CGCATCATTC  
GGACCTGGGT  
ATCCACCCAT  
GTTCCCTACA  
AGAGCGGCTT  
AGTAGTATCT  
GATCATCGAC  
AGATTGCGTT  
GGGTAAACCG  
TGAAACAAAG  
CCGGTATGAG  
CAAAAGAGCA  
GTCAGCATAG  
CTTTAACATG  
AAGGAGGCGAG  
GACCAACAGCC  
CATCGGCAC  
CATCGGGCAC  
CATCGACCCAC  
GTTGCGACAG  
CGAAGGATTTC  
CCCTCGCGGA  
GGTTGTCGCG  
CACCTGGAAT  
CGCAGCACCA  
CCCGCCACCA  
GAACCATGAG  
AAGCAAAAGA  
GTACTGCCAC  
AGCGGACGAT  
AGCAGCAAGC  
AGGCACCATG  
AGGACTACTT  
TAGCAACTCA  
GGAAAAATAT  
TCTGAAAAACA

GACAAAAGCC  
GTGGATCAAC  
CTACCTACGG  
CTTTTGTCA  
AAAACGTCCA  
GACAAAGAAA  
GCAGTCAATG  
ACTTCCACAG  
CTCCCAGCCG  
GCGTGGTTTA  
GTAGACAATA  
TTCCAAGCCA  
TACATTTTAT  
CTCGGCCGCC  
GCGGCCCCGA  
GTAGTGCCCC  
CCGCGGCCCA  
GAGAAGAGA  
AAGTTGGAGG  
GCACTGGCCA  
CCTGTGCTAT  
TTGCCAGTCA  
TATAACTGGC  
GTAGGAGGCA  
ATAGTCTTCG  
AGTAAAGGGA  
CTGGTCACCG  
TGCTATACCC  
GCCTACGATA  
AGCGTCGTTG  
CATACTGAAC  
AACACCATAC  
GCAACAAGT  
GTAGACATCA  
CTCCTCGCAA  
GCAACGTCTC  
GATCTACCTC  
ACTGCAAGGCT

6900

6960

7020

7080

7140

7200

7260

7320

7380

7440

7500

7560

7620

7680

7740

7800

7860

7920

7980

8040

8100

8160

8220

8280

8340

8400

8460

8520

8580

8640

8700

8760

8820

8880

8940

9000

9060

9120

FIG. 3E

ACATCACTAT  
GGAAGTTTA  
ACTACAAGAC  
AGTGCCTCGC  
GACATGACGA  
CCTGCATGGT  
TCCAATTAGA  
AACCAACCAC  
GCCTGGAATA  
CAGGAGACCC  
TGACACCAT  
CAGTGTATG  
ACGCCGTAAT  
CGTTCACCGA  
TGTCATACC  
TTTTAGTGGT  
TTCCAAATGT  
TCAATTTGGA  
TTACCTGCAA  
AATGTCAGCC  
TTATGTGGGG  
ACGTCGAAAT  
CCGCGATGAA  
ACGTGAACGG  
CAGCATCGTT  
ATGACTTCCC  
CCTTGACTAG  
AGAACGTGCA  
CAGGCCGCCC  
GAGCGGTGGA  
TTATCAGGAC  
CTTATTACAG  
AATGCCCCGT  
TGGAGAAAGG  
TATCGCTGTG  
TCGTGAGCAC  
GGAGTTGGCT  
TTTTTGCTTG

GCACAGGGCCG  
CGCAAAGCCG  
CGGAACCGTT  
CTATAAGAGC  
CCACACGGCC  
CCCTGTTGCC  
TACAGACCAC  
TGAATGGATC  
CATATGGGGA  
TCACGGATGG  
CTTAGCCGTC  
TGCTGTAA  
CCCAACTTCG  
GACCATGAGT  
TTTGGCCGCG  
TGCCAGAGAT  
GATCACATGC  
AATCACCACT  
GGCCGCTCAT  
AGGAGCGCAA  
GTCAGCAGAT  
AGTAGGACTG  
AGTCACACCA  
TACGCCATT  
GGAATATGGA  
CAAGGATCTC  
TGTCCTCGTAC  
ACTGCAGGAA  
CTGTTCATAC  
ATCAGATGCA  
AGACTTCGGC  
ACATTCGCAT  
AGCGGTGACA  
TGGGAAGAAG  
CCCGCACAAA  
GTTTGCCCTT  
CAGCATGATG

GGACCGCACG  
CCATCTGGGA  
TCGACCCGCA  
GACCAACGA  
CAAGGAAAT  
CAGCGCCGGA  
TTGACATTGC  
GTCGGAAGA  
AATCATGAGC  
CCACACGAAA  
GCATCAGCTA  
GCGGCCGTG  
CTGGCACTCT  
TACTTGTGGT  
TTCATCGTTC  
TACCTGGCGA  
CCGTATAAGG  
ATGCTCTCGG  
GTGGTCCCT  
GCAGACTATA  
TGTTTTGCG  
TGCGGCTCTG  
CGTATAGTGT  
GGAACGTCTA  
GATCATAGG  
GCGATGAAAC  
ATCGCCAGCA  
ACGCAGGCCG  
ACCGCACCTT  
GGGAACATT  
CCACTGGTCT  
GGATGGCCA  
TCGAGCACAG  
GTACACTTTA  
ACAACATGCA  
AATGACCAAG  
TTGCGCGGCG  
CTGACTAGCA

CTTATACATC  
AGAACATTAC  
CCGAAATCAC  
AGTGGGTCTT  
TGCAATTTGCC  
ATGTAATACA  
TCACCACCAG  
CGGTGAGAA  
CAGTGAGGGT  
TAGTACAGCA  
CCGTGGCGAT  
AGTGCCTGAC  
TGCTGCTGCGT  
CGAACAGTCA  
TAATGCGCTA  
CAGTTGTTGA  
AGTTTTTGCC  
CCCCAAAAT  
CCTGCAAGGT  
ACAGTGAGAA  
ACCAACGCGCA  
ACGGGAACAC  
AAGACTTGAA  
TCGTTATCCA  
CAGGAGCGTT  
CAGACATTAG  
CATCAGGATT  
TCGGGTGTAA  
CCAATTTCTAT  
CAACAGTCAA  
CCCTGCAGTA  
CAACTCTCCA  
GCACCGCGAG  
ATGCAGAAATG  
AATTTCAAGC  
CCTCGTCGCT  
CAGCAAGATG

CTACCTGGAA  
GTATGAGTGC  
TGGTIGCACC  
CAACTCACCG  
TTTCAAGTTG  
TGGCTTTAAA  
GAGACTAGGG  
CTTACCCGTC  
CTATGCCCAA  
TTACTACCA  
GATGATTGGC  
GCCATACGCC  
TAGGTGCGCC  
GCCGTTCTTC  
CTGCTCCTGC  
CTACGAACT  
AAGGGCAGGG  
TTCCACCAAC  
CAAAATGCTGC  
CTTCGGAGGG  
CAGCCAGATG  
GGCGATTAA  
TACCAATTTC  
AGTCAATAGCT  
TCGCGGCCCTG  
CGGAGACATT  
GCTACTCAAG  
TGAGATGTGG  
GATTGCACTA  
TGACATCCCG  
ATGTGAAGTC  
TGATCCGAC  
AGATCCGAC  
TCCACAGGCA  
TAAACACCA  
CGCCATCTCA  
ATTAAATTATA  
ACCGCTACGC

GAATCATCAG  
AAGTGGGCG  
GCCATCAAGC  
GACTTGATCA  
ATCCCAGGTG  
CACATCAGCC  
GCAAAACCCG  
GACCGAGATG  
GAGTCAGCAC  
CGCATCCTG  
GTAACTGTTG  
CTGGCCCCAA  
AATGCTGAAA  
TGGTCCAGT  
TGCTGCCTT  
GCGACCACTG  
TATGCCCCGC  
CAAGAGTACA  
GGCTCCTGG  
GTCTACCCCT  
AGTGAGGCGT  
GTGCACTAG  
CTAGATGTGT  
GGACCAATTT  
GTGTACAAT  
CAAGCTACCT  
CCTTCGCCA  
AAAACAAT  
AATCCGCTCC  
AACGCTGCC  
AGTGAGTGCA  
GCGAAGGTCT  
GTACATGTCC  
AATCTTATCG  
GCTGACCAT  
AAAACATCAT  
GGACTTATGA  
CCCAATGATC

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11100  
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11400

FIG. 3F

CGACCAAGCAA  
CAATTAGATCC  
GGAAGCGCAG  
CTAGCGGACG  
CGTCTGCATA  
AAAAAATAA  
CTAGAGCTCG  
CCTCCCGCTG  
ATGAGGAAAT  
GGCAGGACAG  
GCTCTATGGC  
CCTGTAGCGG  
TTGCCAGCGC  
CCGGCTTTCC  
TACGGCACCT  
CCTGATAGAC  
TGTCCCAAC  
TTTTGGGAT  
ATTAATTCG  
GCAGAAGTAT  
GCTCCCGCAG  
CGCCCCTAAC  
ATGGCTGACT  
TCCAGAAGTA  
CTTGATATC  
ACAAGATGG  
ACTGGGCACA  
GGCGCCCGGT  
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TGTCACTCA  
TGCAATACGT  
GAGCACGTAC  
AGGGGCTCGC  
ATCTCGTCGT  
TTTCTGGATT  
TGGCTACCCG  
TTTACGGTAT

AACTCGATGT  
CCGCTTACC  
TGCAATAATGC  
CCAAAACTC  
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TGCAATCGAT  
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CCGTCAAGCT  
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TGGAAACAAC  
TTCGGCTAT  
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ATTGACGGA  
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TCTTTTGTG  
GCTATCGTGG  
AGCGGGAAGG  
CCTTGTCTCT  
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TCGGATGGAA  
GCCAGCCGAA  
GACCCATGGC  
CATCGACTGT  
TGATATTGCT  
CGCCGCTCCC

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TCGACTGTGC  
ACCTTGGAG  
TGCTTGAGT  
GATTGGGAG  
GAAAGAACCA  
GCGGCGGTG  
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AAACTTGATT  
CCTTTGACGT  
CTCAACCCCTA  
TGGTTAAAA  
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CATCTCAATT  
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CCGCCCTTAA  
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TTTTTTGGAG  
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GGCTGCTCTG  
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GATGCCTGCT  
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GATTCGCAGC

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TGCCACATAA  
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CTTCTTTCCC  
GCATCCCCTT  
AGGGTGATGG  
TGGAGTCCAC  
TCTCGGTCTA  
ATGAGCTGAT  
GTGTGGAAAG  
AGTCAGCAAC  
TGCACTCTCA  
CTCCGGCCAG  
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GCCTAGGCTT  
AGCAGGATG  
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CGGGCGTTCC  
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TGCCGAATAT  
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AGGGTTCCGA  
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TTCTTTTGTAT  
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TCCCCAGGT  
CAGGTGTGGA  
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TTCCGGCCCAT  
CGCCCTCTGC  
TTGCCAAAAG  
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AGGATCGTTT  
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GTTCCGGCTG  
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AGTGCCGGGG  
GGCTGATGCA  
AGCGAAACAT  
TGATCTGGAC  
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GCGCATGCC  
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CAGGCTGGTA  
GTACTTCCGA  
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GGGCCATCGC  
AGTGGACTCT  
TTATAAGGGA  
TTTAAAGGCA  
CCCCAGGCG  
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ACCATAGTCC  
TCTCCGCCCC  
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CTCCGGGAG  
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TTCGGCTATG  
TCAGCGCAGG  
CTGCAGGACG  
GTGCTCGACG  
CAGGATCTCC  
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CGCATCGAGC  
GAAGAGCATC  
GACGGCGAGG  
AATGGCCGCT  
GACATAGCGT  
TTCTCGTGC  
CTTGACGAGT

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**FIG. 3G**

13740	ACCTGCCATC	GCGACGCCCA	GACCGACCAA	GGTTCGAAAT	GGACTCTGG	GGTCTCTGAGC
13800	TCGTTTTCCG	GGCTTCGGAA	TGAAAGGTTG	CCGCTTCTTA	GATTCACCG	GATTCACGAGA
13860	TCGCCCAACC	CTGGAGTTCT	GGATCTCATG	TCCAGCGCGG	TGGATGATCC	GGGACGACCCGGC
13920	CAAAATTCAC	AATAGCATCA	CAAAATAAGC	ATAATGGTTA	ATTGCAGCTT	ATTGCAGCTT
13980	TCAATGATATC	TCAAAATCTA	TTGTGGTTTG	TGCATCTAG	TTTTTTTAC	TTTTTTTAC
14040	GGTCAATAGCT	GCCTAAATCAT	CTAGAGCTTG	CGACCTCTAG	TGTATACCGT	TTTTTATCATGTC
14100	CGGAAGCAT	AACATACGAG	AAATCCACAC	ATCCGCTCAC	TGAAATGTT	TTTTTCTGTG
14160	CGTTGCGCTC	ACATTAATTTG	GAGCTAACTC	CCTAATGAGT	GCCTGGGGTG	TTTTTAAAGTGTA
14220	TCGGCCCAACG	CATTAATGAA	GTGCCAGCTG	GAAACCTGTG	TTCCAGTCCG	TTCCAGTCCG
14280	CTGACTCGCT	TCCTCGCTCA	CTCTTCGGCT	GTAATGGCG	GGCGGTTTGC	GGCGGTTTGC
14340	TAATACGGTT	TCAAAGGCGG	ATCAGCTCAC	GGCAGCGGTT	GTTCGGCTGC	GTTCGGCTGC
14400	AGCAAAGGCC	GCAAATGTGA	GAACATGTGA	ACGCAGGAAA	TCAGGGGATA	TCAGGGGATA
14460	CCCCACGACA	AGGCTCCGCC	GTITTTCCAT	CGTTGCTGGC	AAATAAGGCCG	AAATAAGGCCG
14520	TATAAAGATA	CGCAGCAGGAC	GTGGCGAAAC	CAAGTCAGAG	AATCGACGCT	AATCGACGCT
14580	TGCCGCTTAC	GTTCCGAGCC	GGCGTCTCTT	GCTCCTCTGT	CCCCCTGGAA	CCCCCTGGAA
14640	GCTCACGGTG	CTTTCTCAAT	AAGCGTGGCG	TCCCTTCGGG	TCCGCTTTTC	TCCGCTTTTC
14700	ACGAACCCCC	GGCTGTGTGC	CTCCAAGCTG	AGGTCGTTG	AGTTCGGTGT	AGTTCGGTGT
14760	ACCCGGTAAG	CTTGAGTCCA	TAACATCTGT	CTTATCCGG	GACCGCTGCG	GACCGCTGCG
14820	CGAGGATAGT	ATTAGCAGAG	TGGTAACTAG	CAGCAGGCCAC	TCGCCACTGG	TCGCCACTGG
14880	GAAGGACATG	GCTACACATA	GCCTAACTAG	TGAAGTGGTG	ACAGAGTTCT	ACAGAGTTCT
14940	GTAGCTCTTG	AAAAGAGTTG	TACCTTCGGA	TGAAGCCAGT	TGCGCTCTGC	TGCGCTCTGC
15000	AGCAGATTAC	GTTTGCAAGC	TGGTTTTTTT	CTGGTAGCGG	CAAAACACCG	CAAAACACCG
15060	CTGACGCTCA	TCTACGGGGT	TTTGATCTTT	AAGAAGATCC	AAAGGATCTC	AAAGGATCTC
15120	GGATCTTCAC	TTATCAAAAA	GGTCATGAGA	AAGGGA	AACACACETT	AACACACETT
15180	ATGAGTAAAC	TAAAGTATAT	TAAATCAATC	AATGAAGTTT	TTAAATTA	TTAAATTA
15240	TCGTCTATT	ATCTCAGCGA	TGAGGCACCT	GCTTAATCAG	AGTTACCAAT	AGTTACCAAT
15300	GGGAGGGCTT	ACTACGATAC	CGTGATAGTA	GACTCCCGT	ATAGTTGCCT	ATAGTTGCCT
15360	CTCCAGATTT	CCTCACCGG	CGCAGACCCA	CAATGATACC	CCCAGTGCCTG	CCCAGTGCCTG
15420	CAACTTTTATC	AGTGGTCTTG	CGAGCGCAGA	CCGGAAGGGC	AACGAGCCAG	AACGAGCCAG
15480	CGCCAGTTAA	GTAAGTAGTT	GGAAGCTAGA	ATTGTTGCCG	CAGTCTATTA	CAGTCTATTA
15540	CGTCGTTTGG	GGTGCACGCT	AGGCATCGTG	CCAATTGCTAC	AACGTTGTTG	AACGTTGTTG
15600	CCCCCATGTT	GTTACATGAT	ATCAAGGCCGA	GTTCCTCAACG	TTACAGTCCG	TTACAGTCCG
15660	AGTTGGCCCG	CTCAGAAGTA	TCCGATCTGT	CCTTCGGTCC	GCGGTTAGCT	GCGGTTAGCT
15720	TGCCATCCGT	TTCTACTGTCA	GCATAATCTT	TGGCAGCACT	CTCATGGTTA	CTCATGGTTA
15780	AGTGATGCG	TCTTGAGAA	ACCAAGTCA	GTGAGTACTC	TCTGTGACTG	TCTGTGACTG
15840	ATAGCAGAAC	ACCGGCCAC	ACGGATAAT	CGGCGTCAAT	TGCTCTTGCC	TGCTCTTGCC
15900	GGATCTTACC	AAACTCTCAA	TTCGGGGCCGA	GAAAACGTTT	CTCATCATTTG	CTCATCATTTG
15960	CAGCATCTTT	AACATGATCTT	TCGTGACCC	TGTAACCCAC	TCCAGTTCGA	TCCAGTTCGA

FIG. 3H

TACTTTCACC AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAATGCCG 16020  
AATAAGGCG ACACGGAAAT GTTGAACTACT CTTTTCAT GATTTGAAG 16080  
CATTATCAG GGTATTGTC TCATGAGCGG ATACATATTT GAATGTAAT 16140  
ACAAATAGGG GTTCCGCGCA CATTCCCCCG CTTGAGCTA AGGATCGGG 16200  
AGATCTAATG AAGACCCCA CTTGTAGTT AAGCAAGTTC TTTAAGTAA 16260  
CAAGGCATGG AAAATACAT AACTGAGAA TGGCAAGCTA GATCAAGGT 16320  
TGGAACAGCT GAATATGGC CAAACAGGAT ATCTGTGGTA AGCATTCCT 16380  
AGGGCCAAGA ACAGATGGAA CAGCTGAATA TGGGCCAAAC AGGATATCTG 16440  
TTCCTGCCCC GGCTCAGGGC CAAGAACAGA CAAGTCCCCAG ATGCGGTCCA 16500  
GTTTCTAGAG AACCATCAGA TGTTCCAGG GTGCCCCCAAG GACCTGAAAT 16560  
CTTATTTGAA CTAACCAATC AGTTCGCTTC TCGCTTCTGT TCGCGCGCTT 16620  
AGCTCAATAA AAGAGCCAC AACCCCTCAC TCGGGG 16656

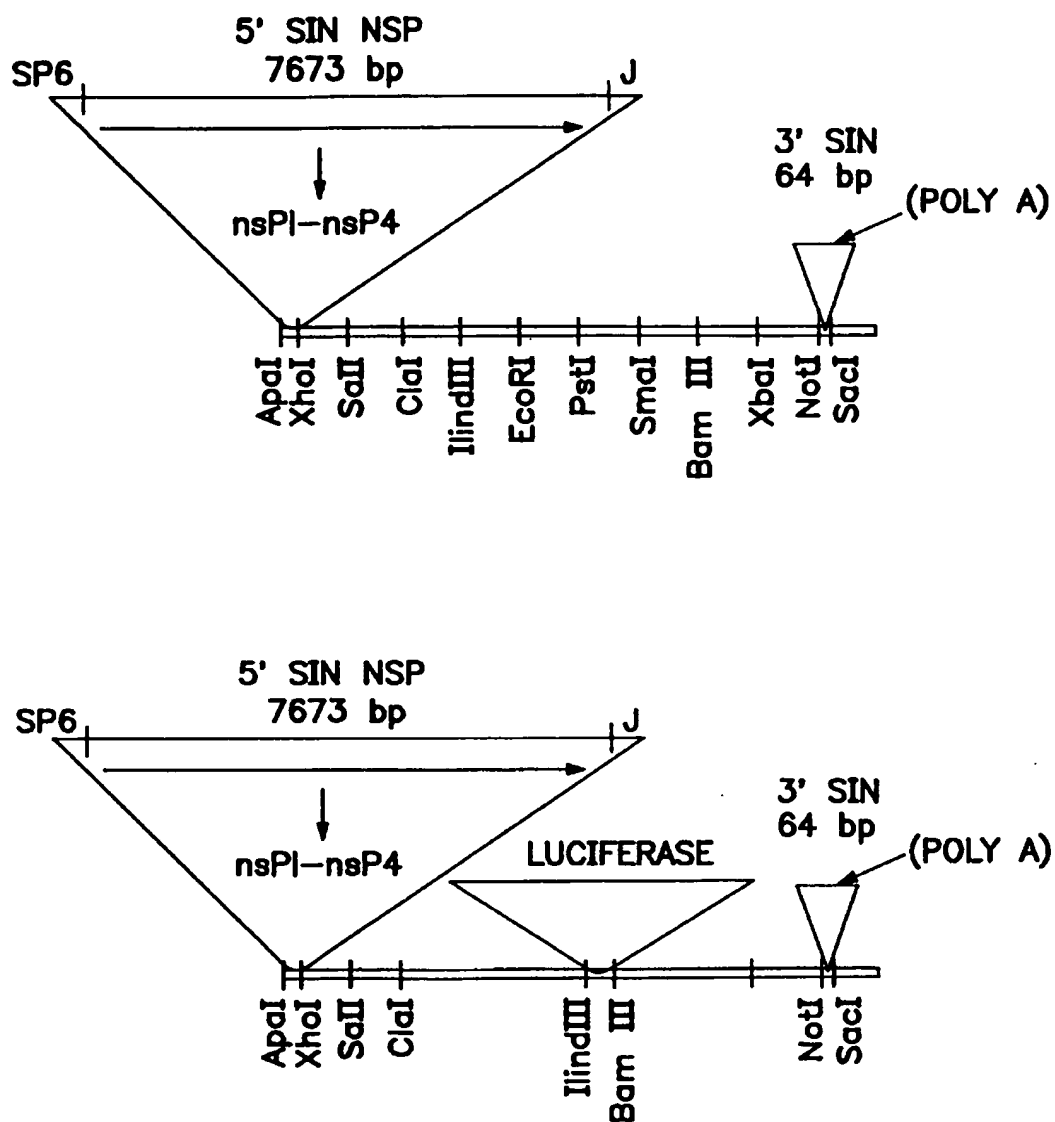


FIG. 4

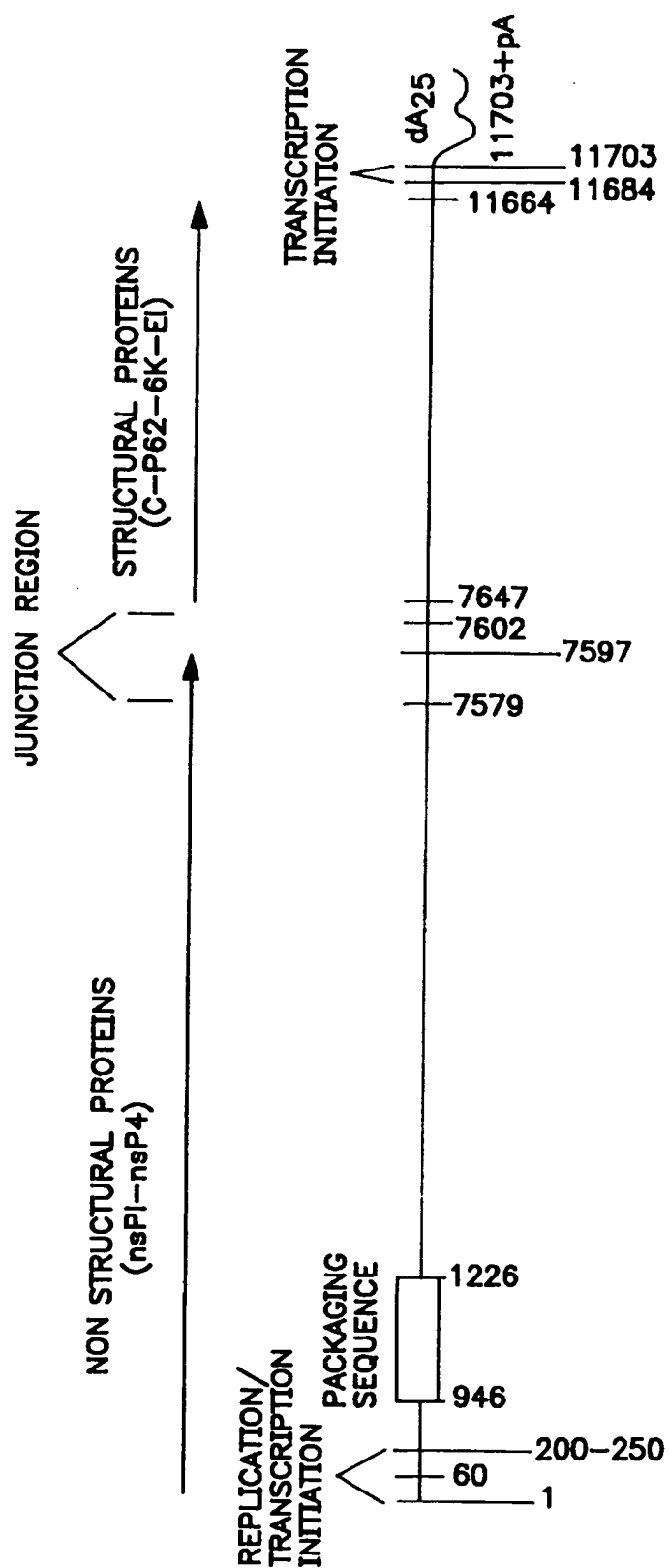


FIG. 5A



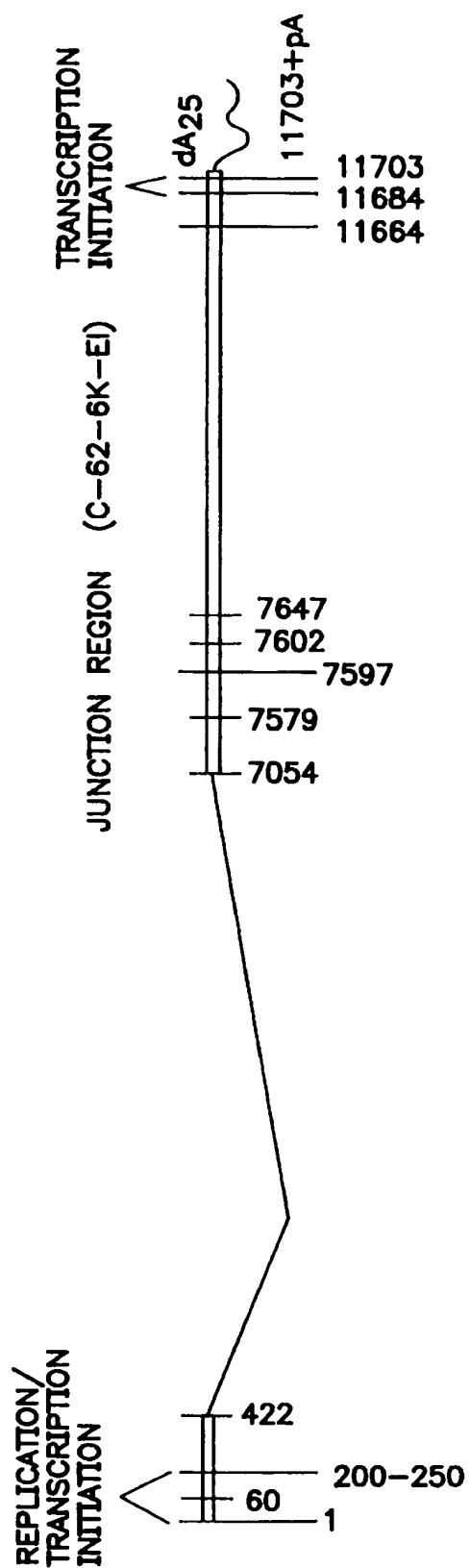


FIG. 5B

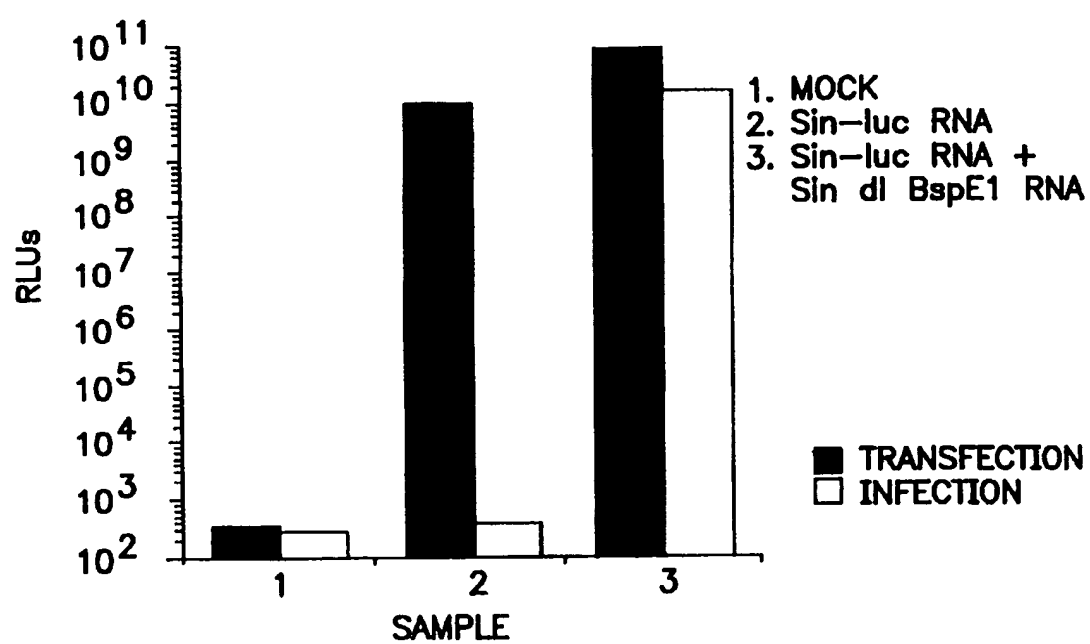


FIG. 6

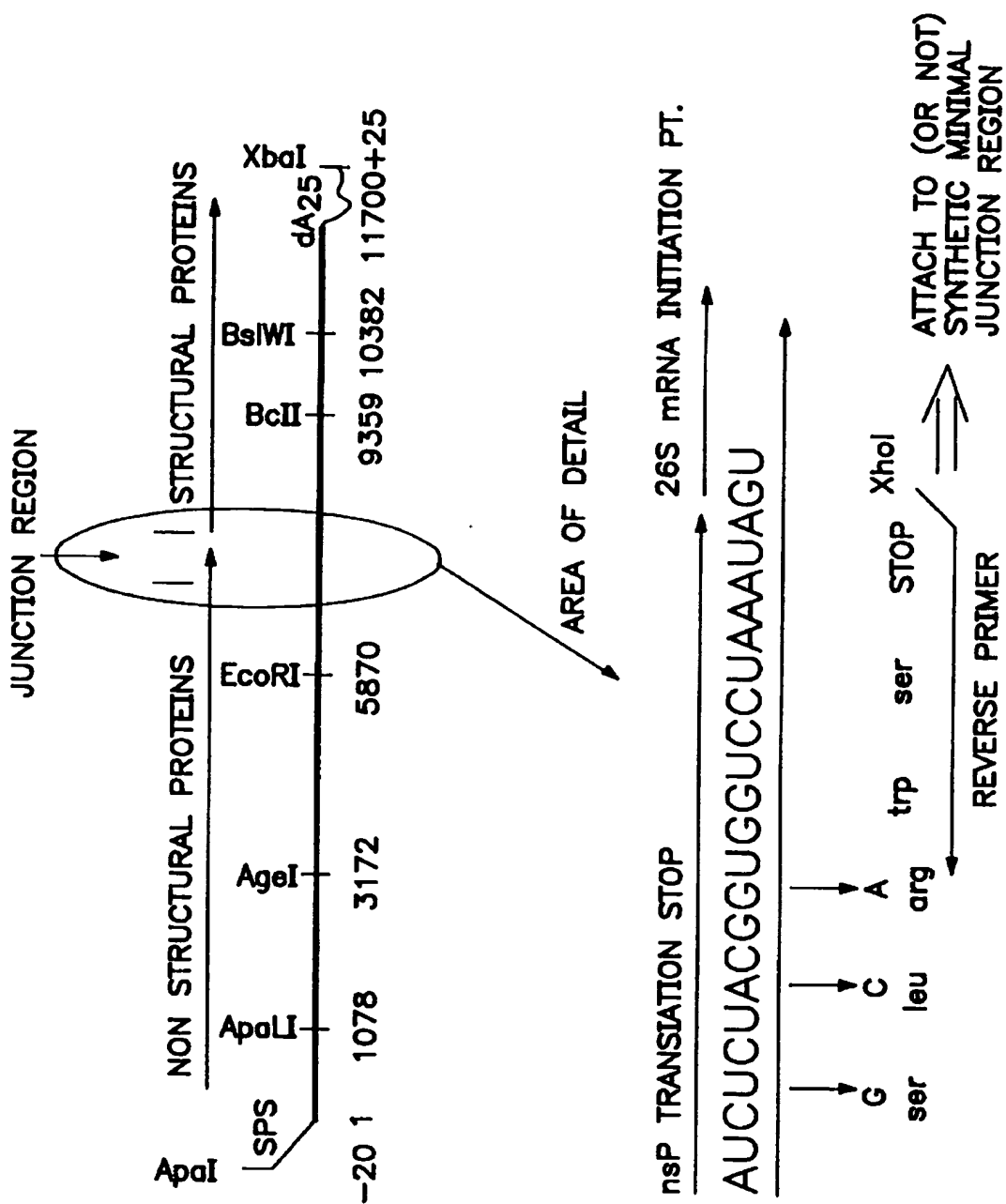


FIG. 7

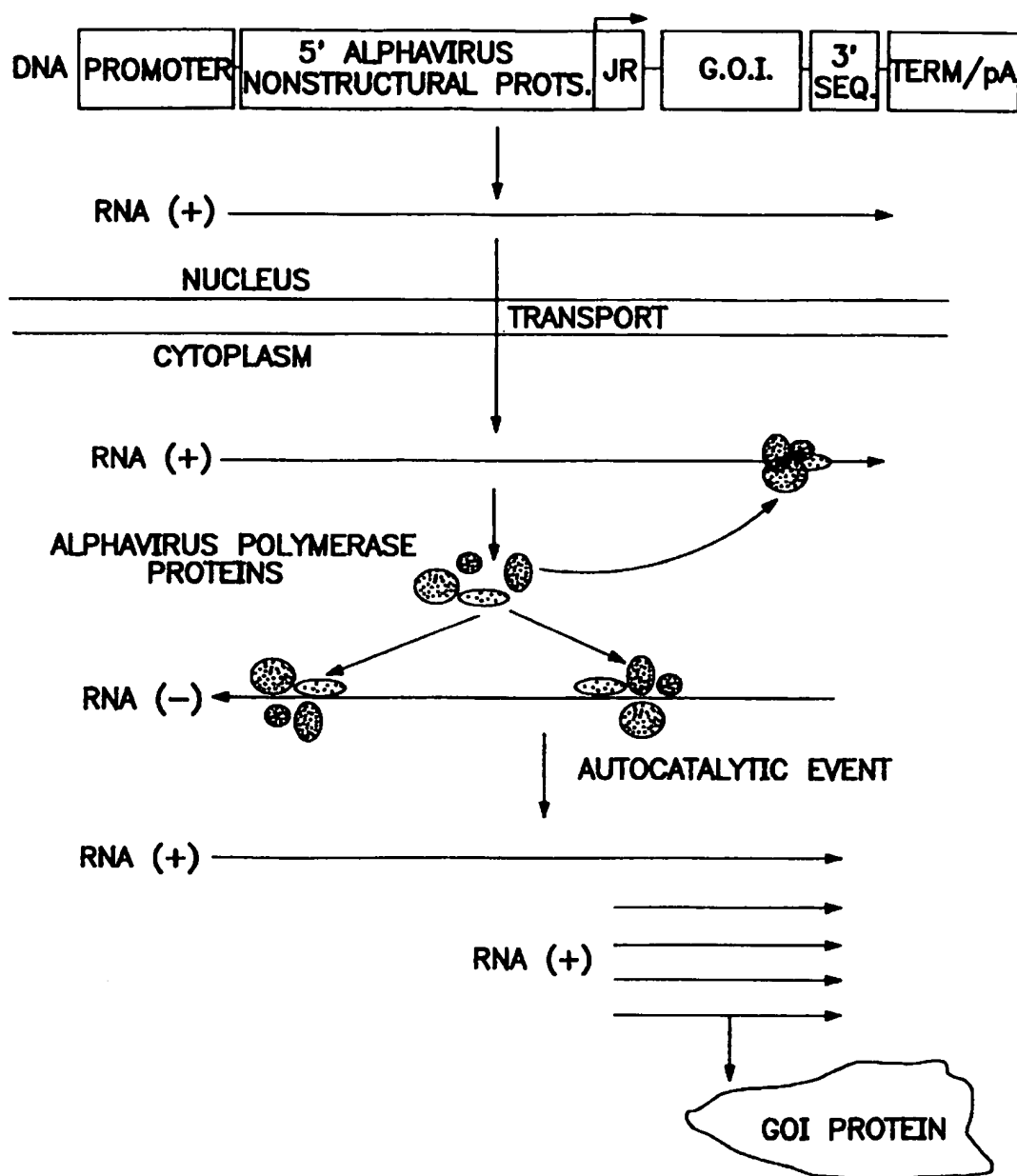


FIG. 8

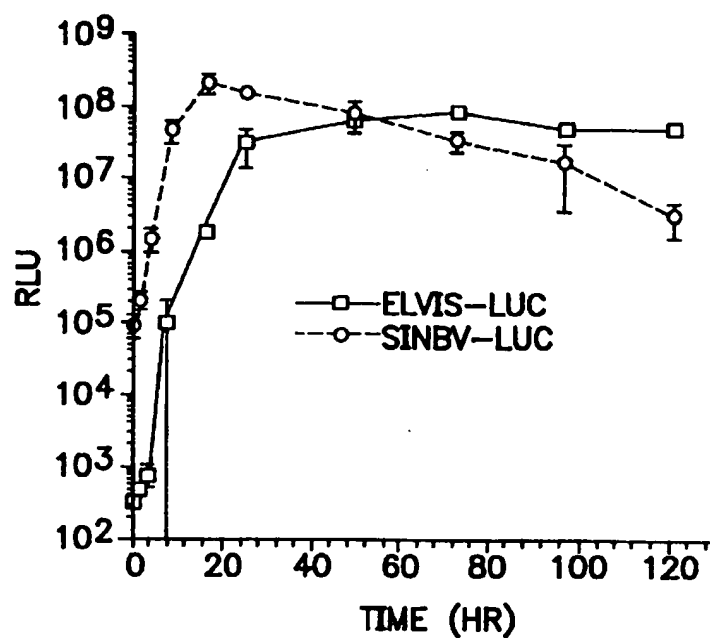


FIG. 9

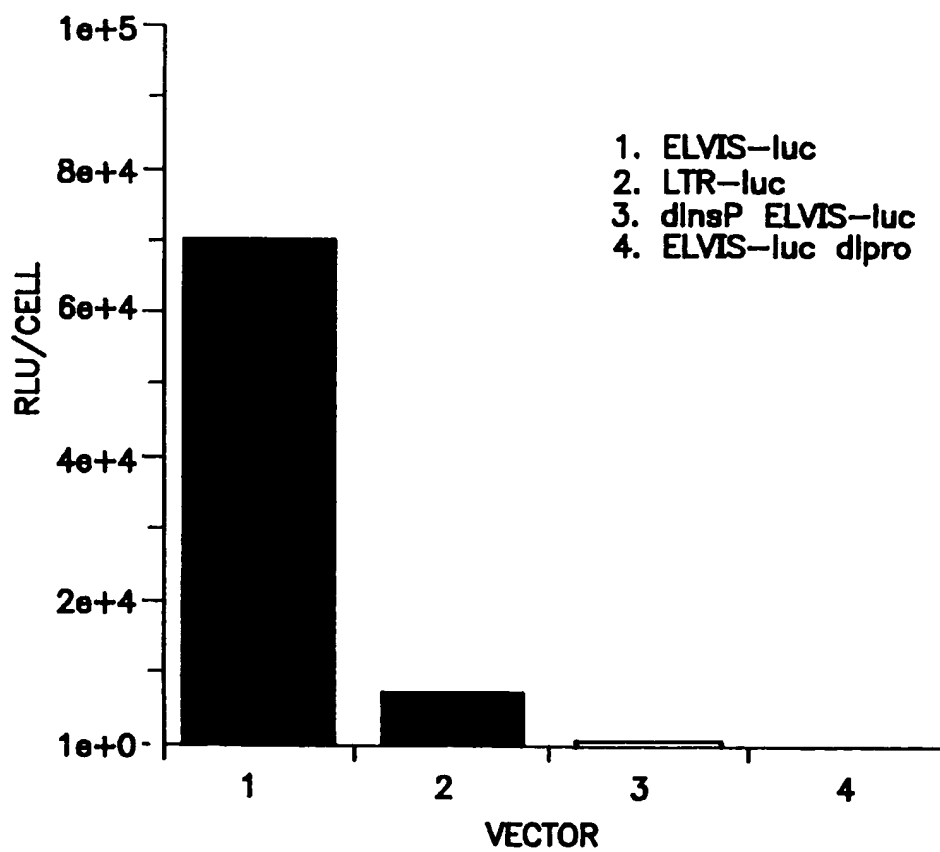


FIG. 10

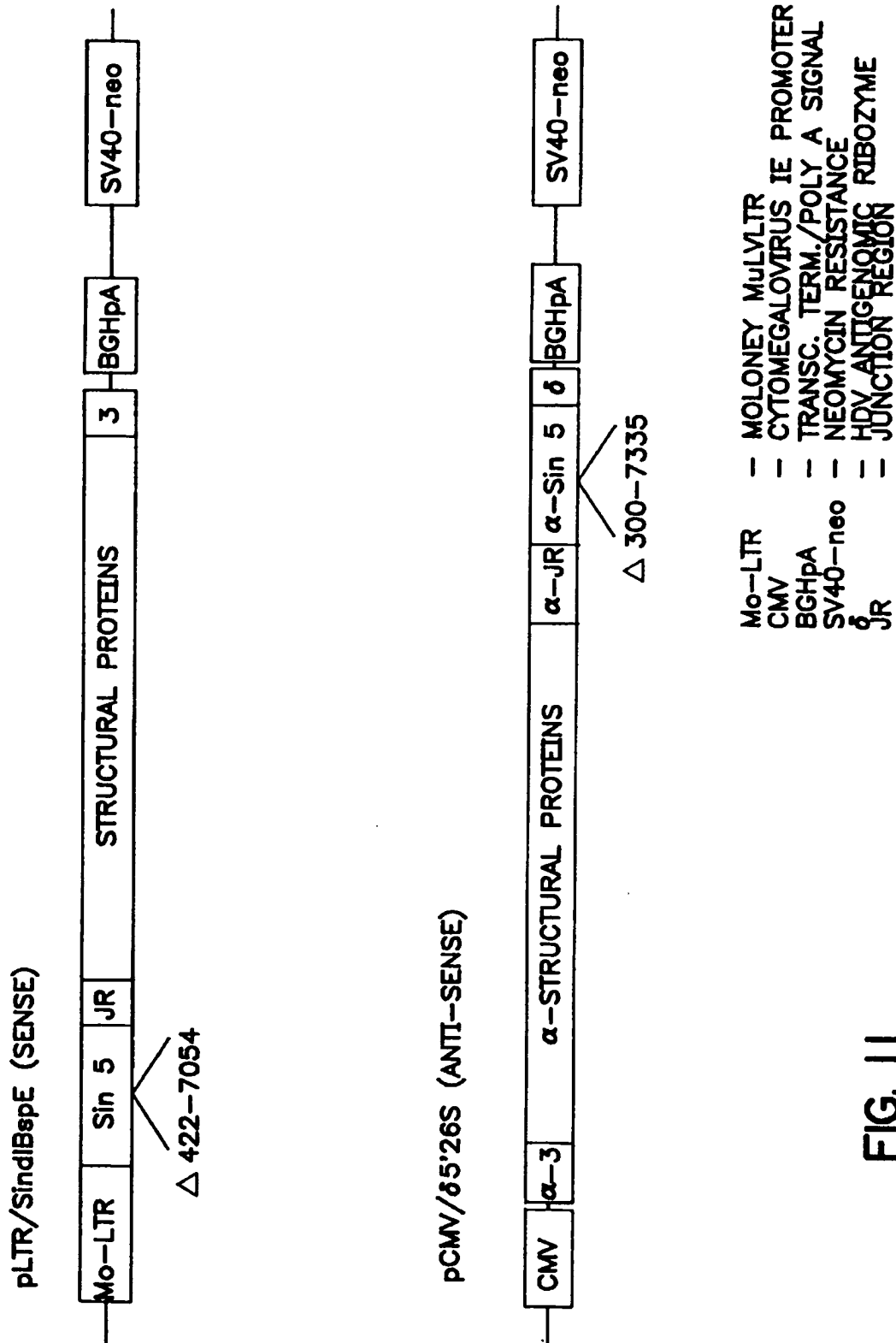


FIG. 11

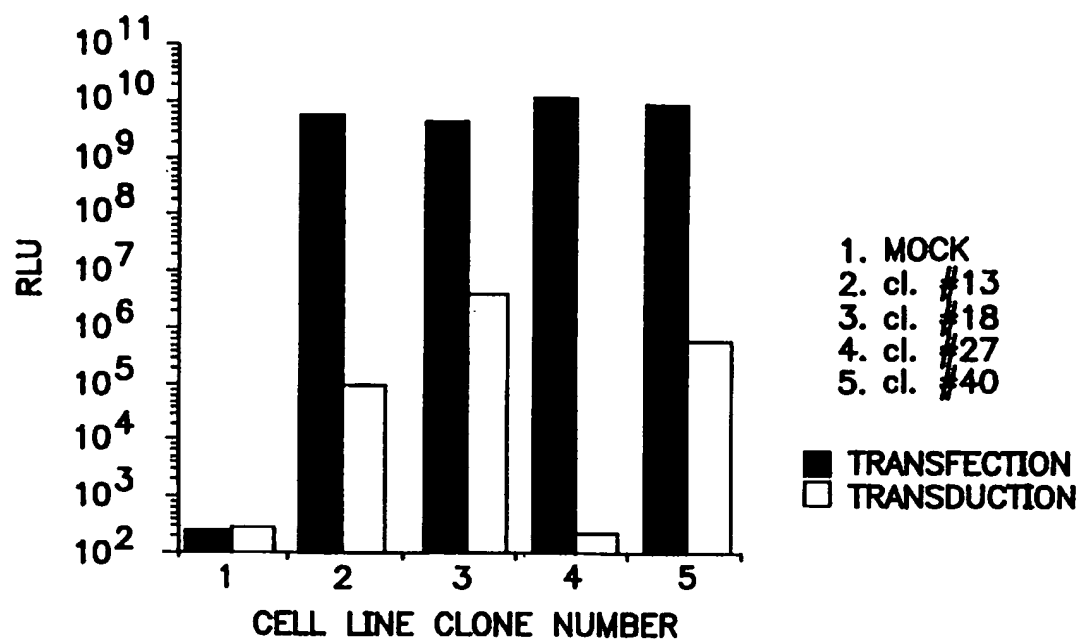


FIG. 12

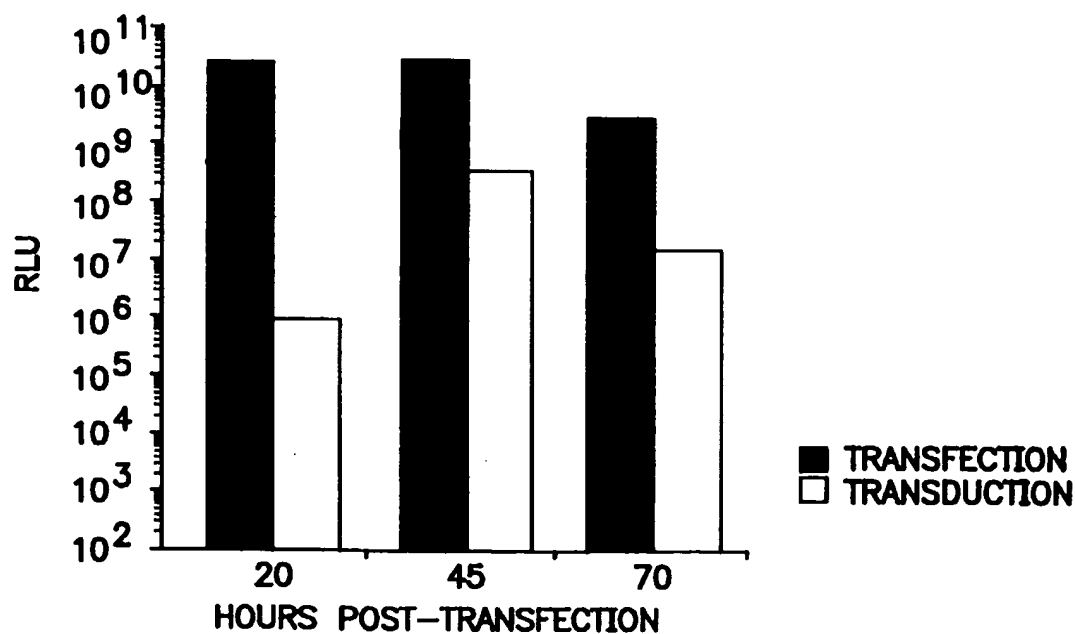


FIG. 13

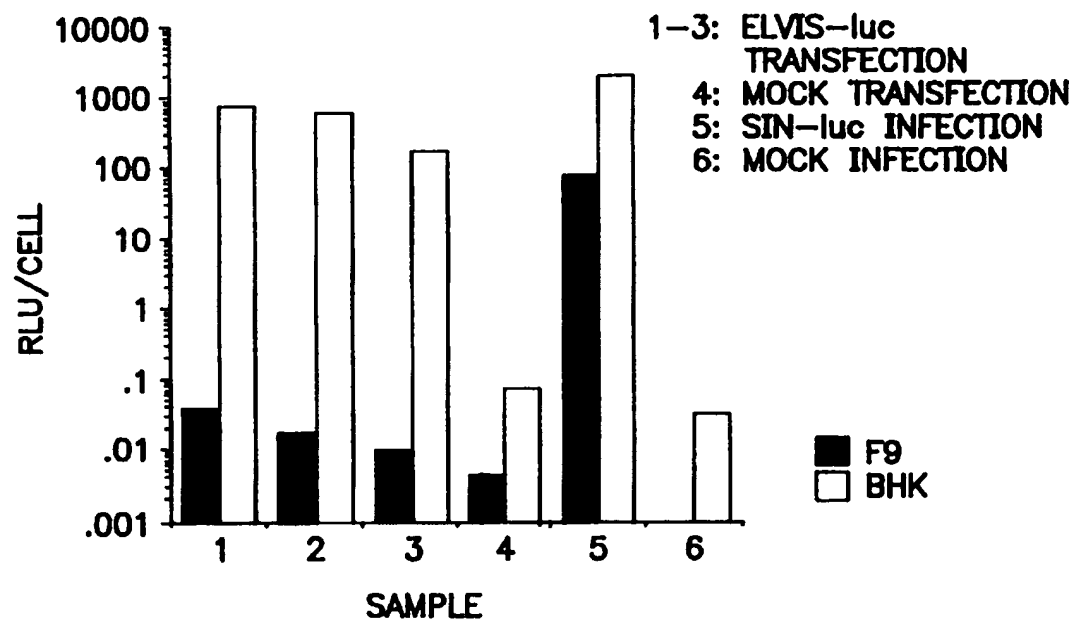


FIG. 14



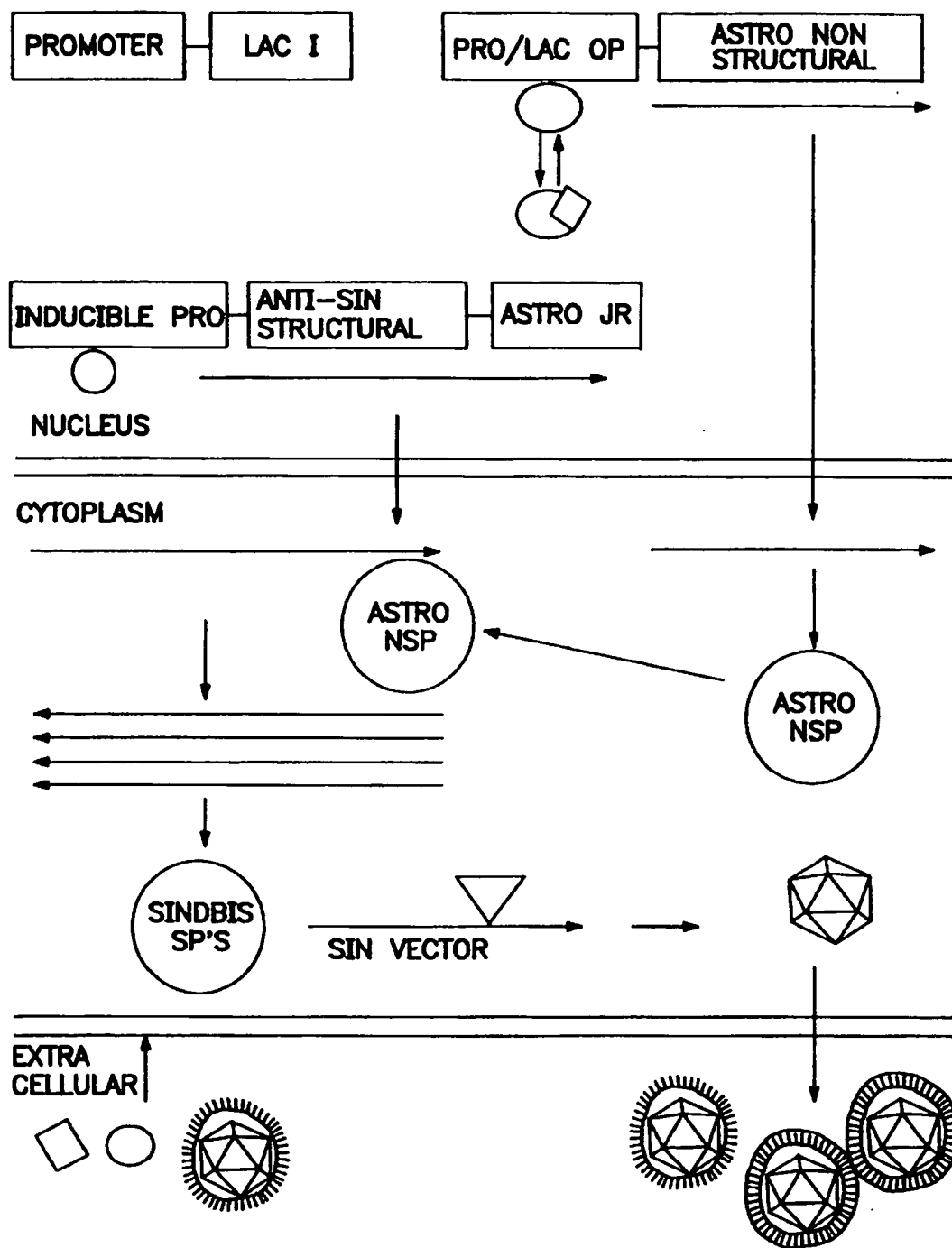


FIG. 15

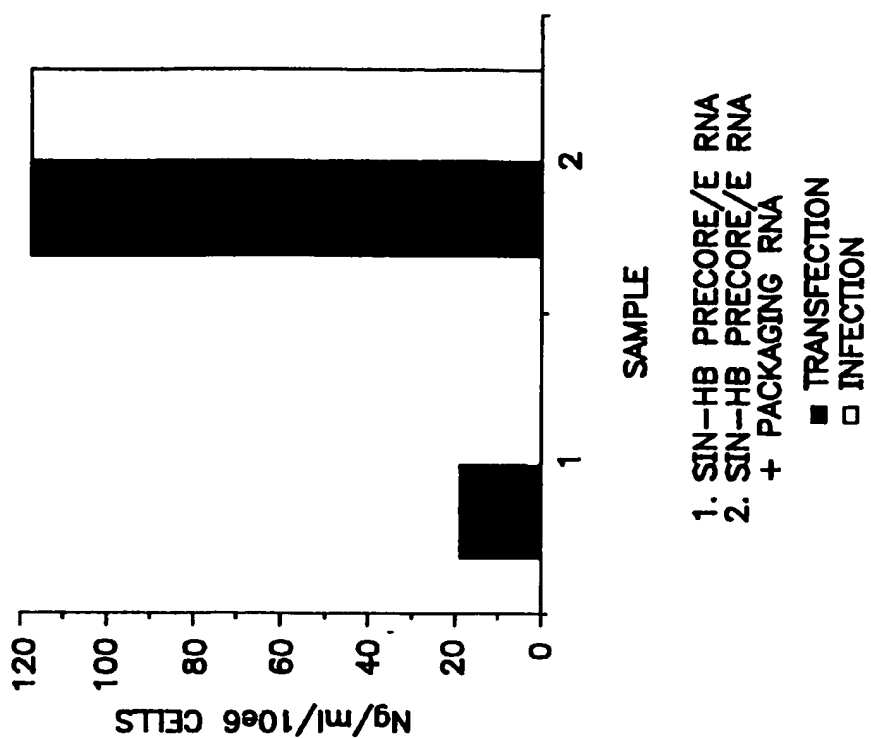
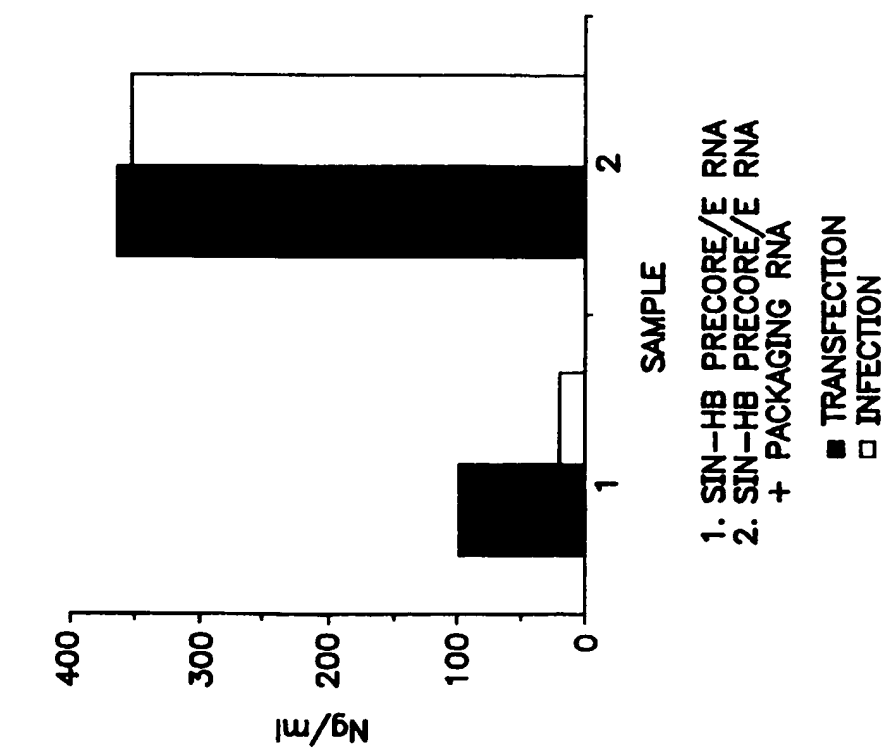


FIG. 16B



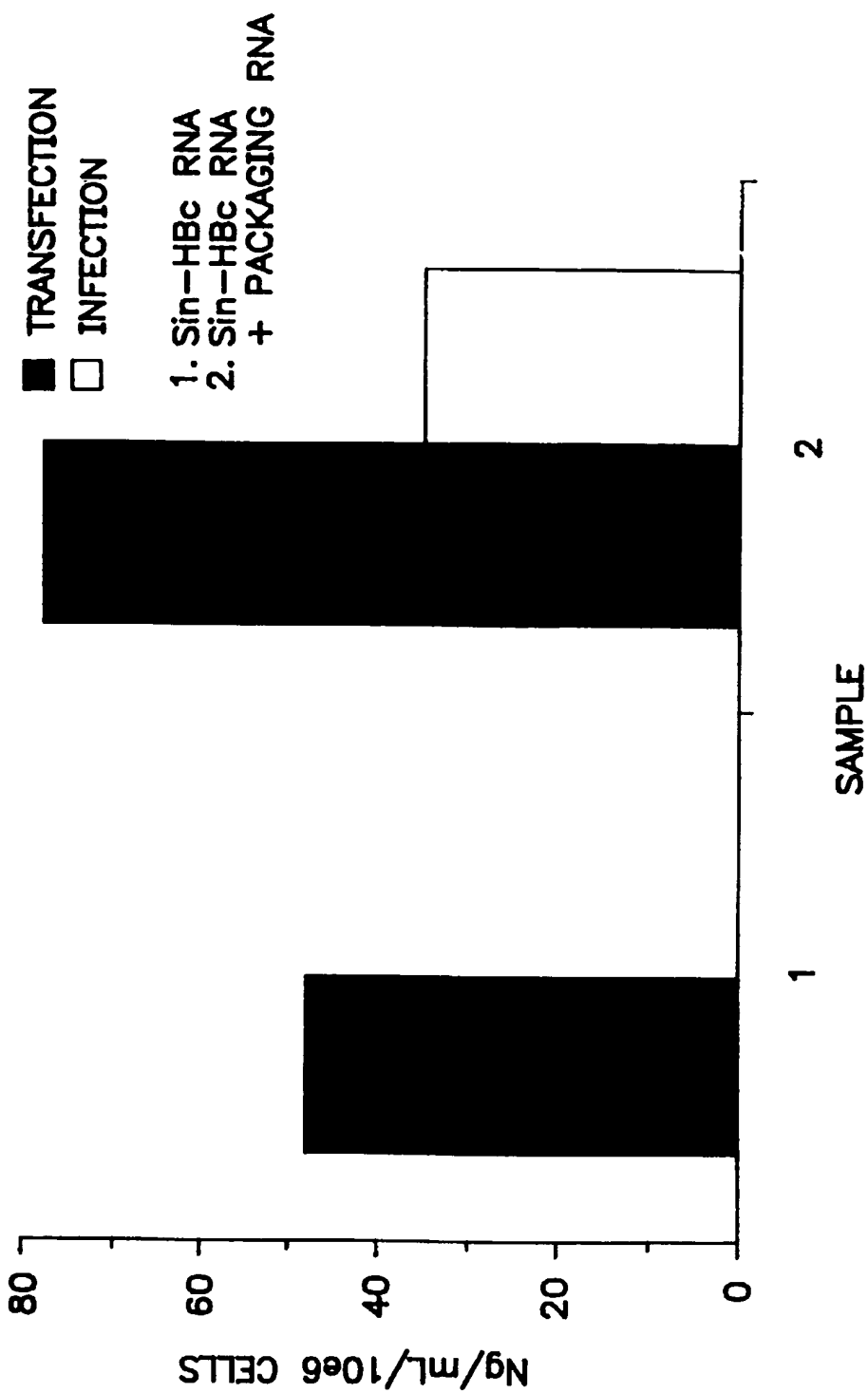


FIG. 17

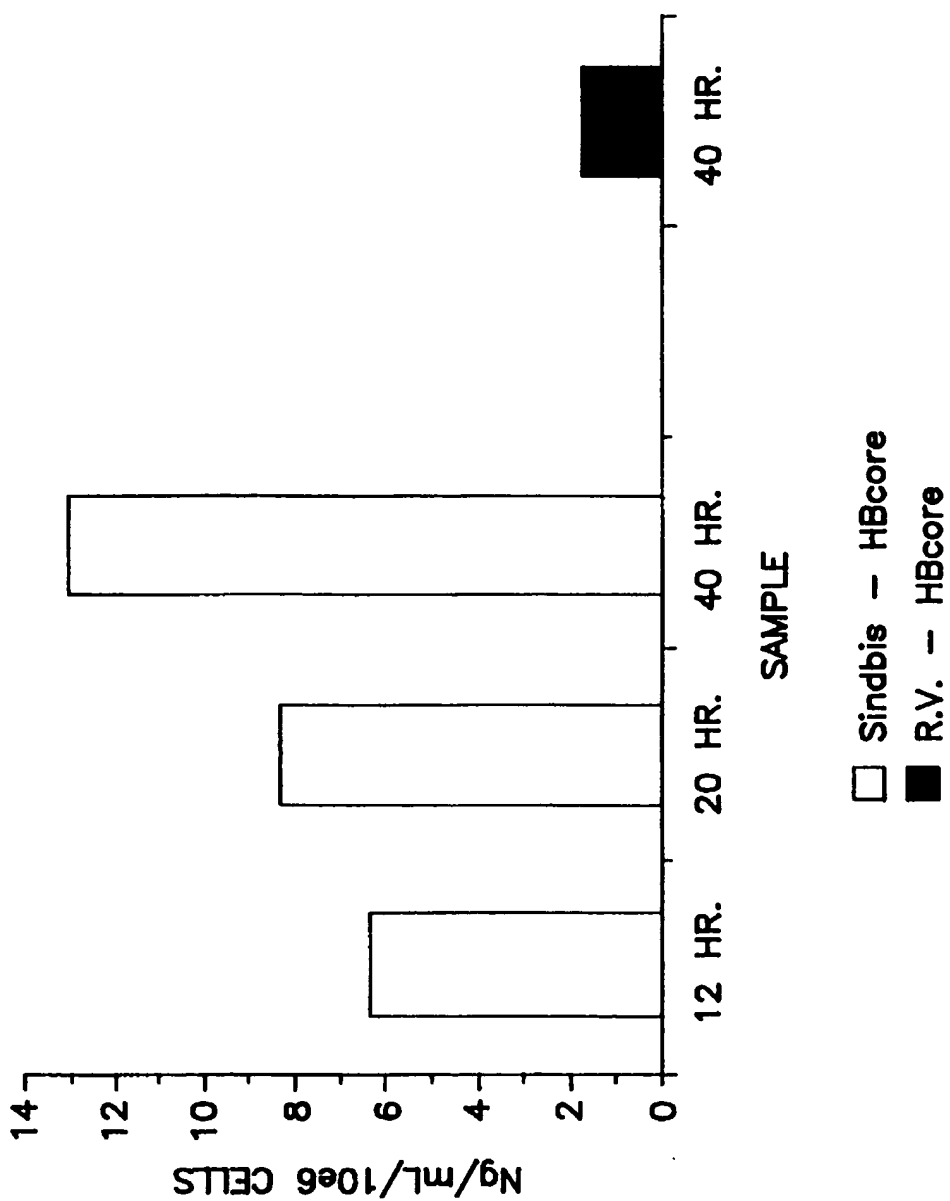


FIG. 18

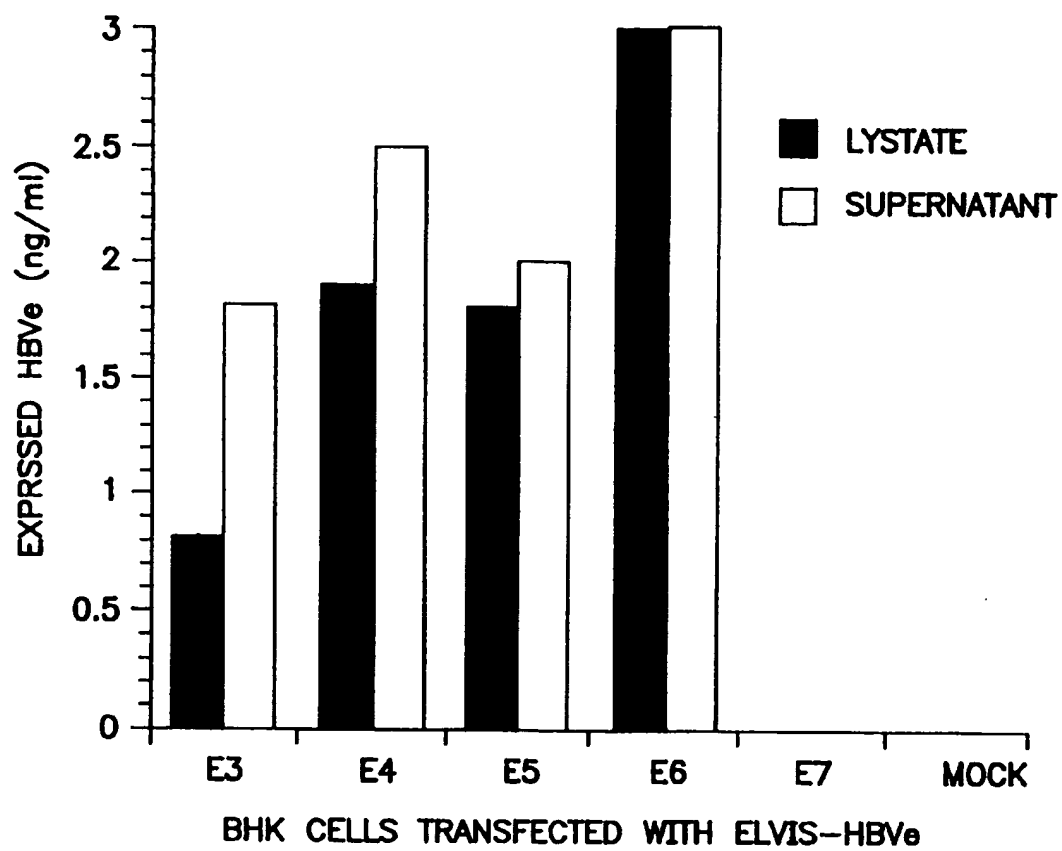
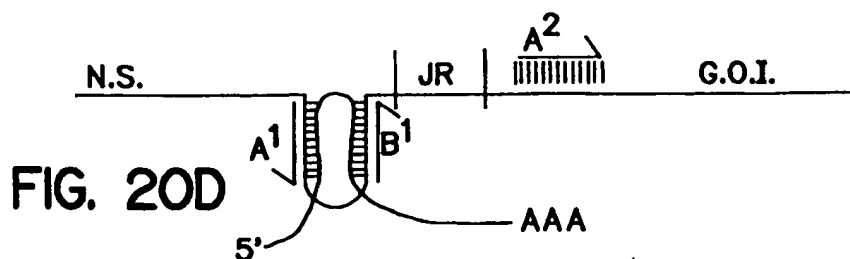
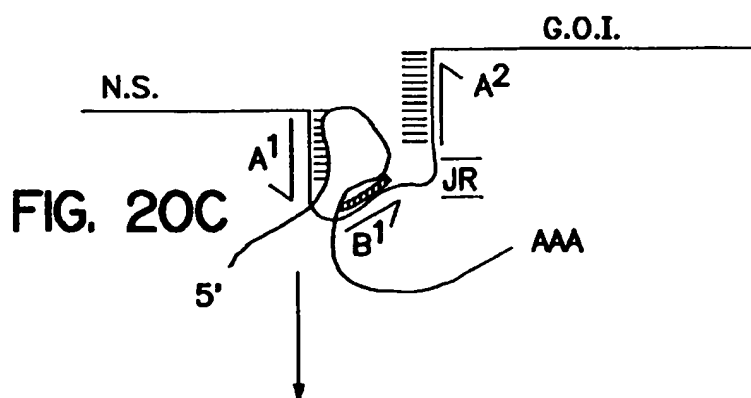
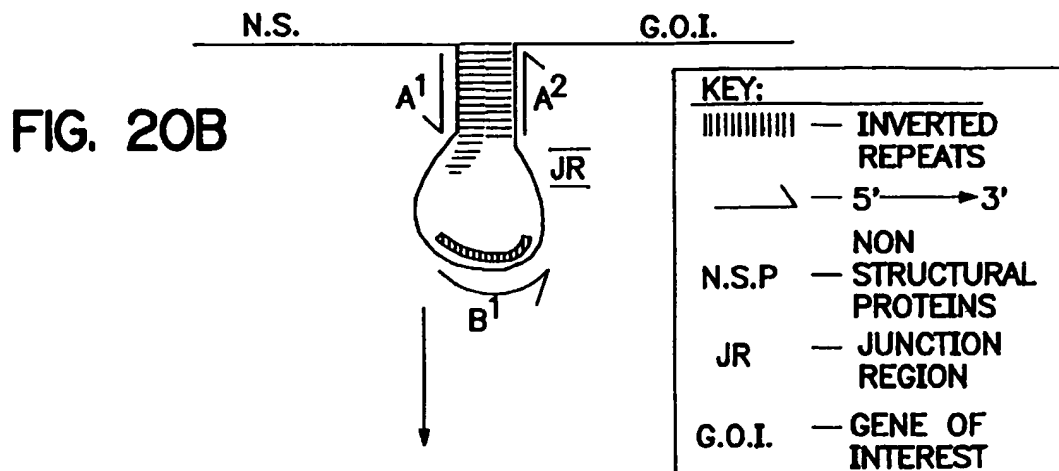
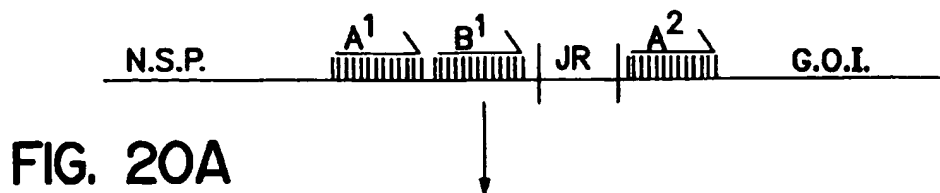


FIG. 19



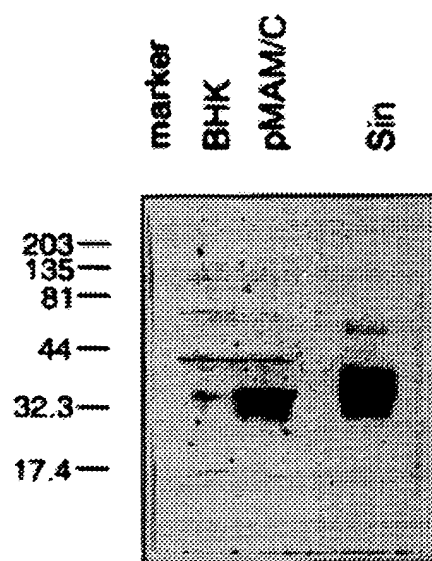


FIG. 21

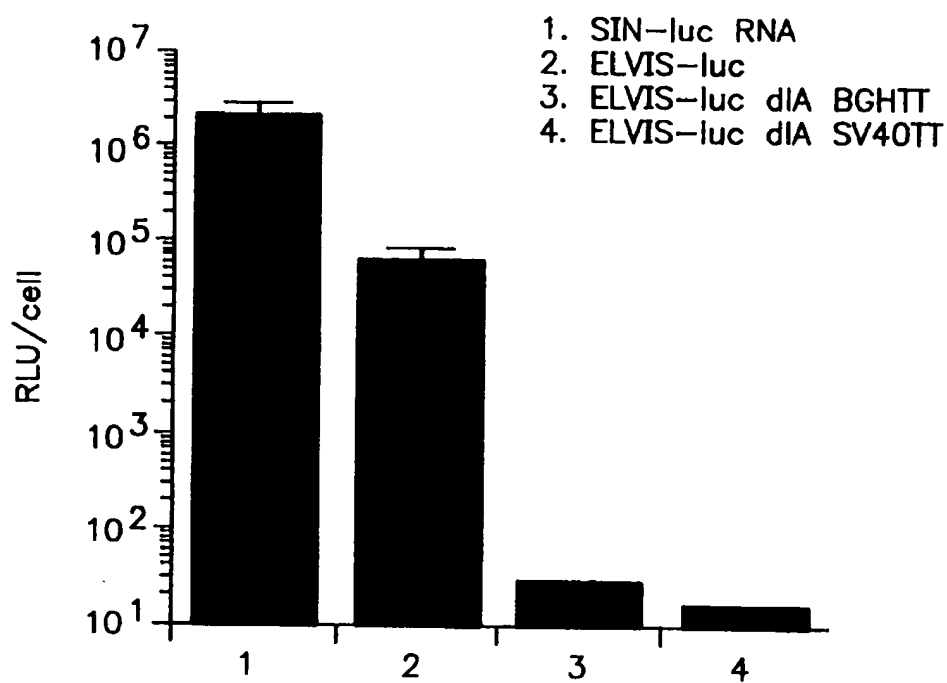


FIG. 22A

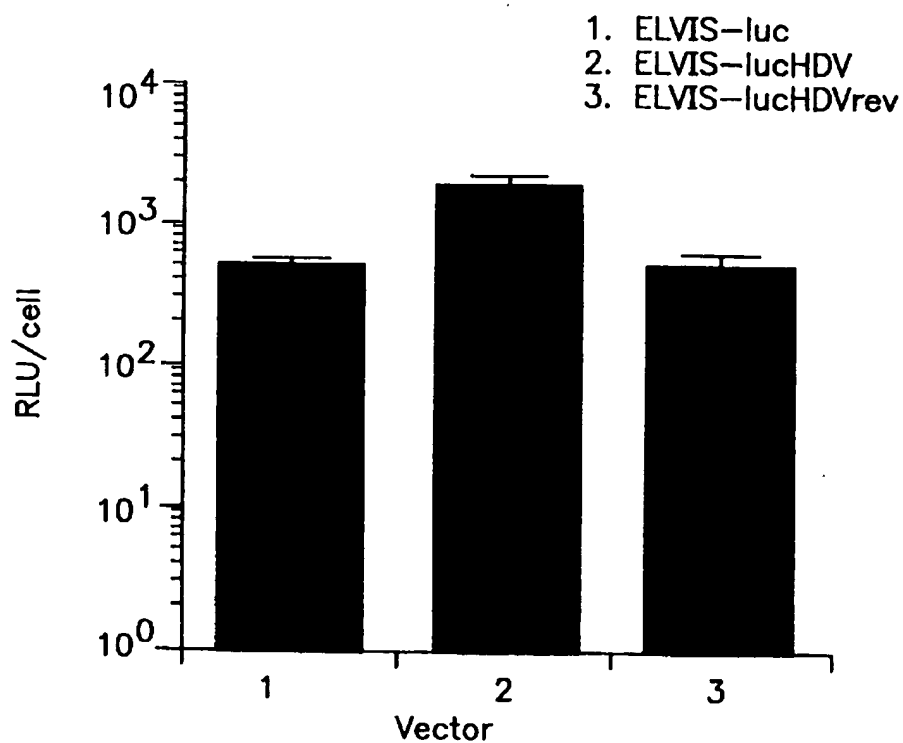


FIG. 22B



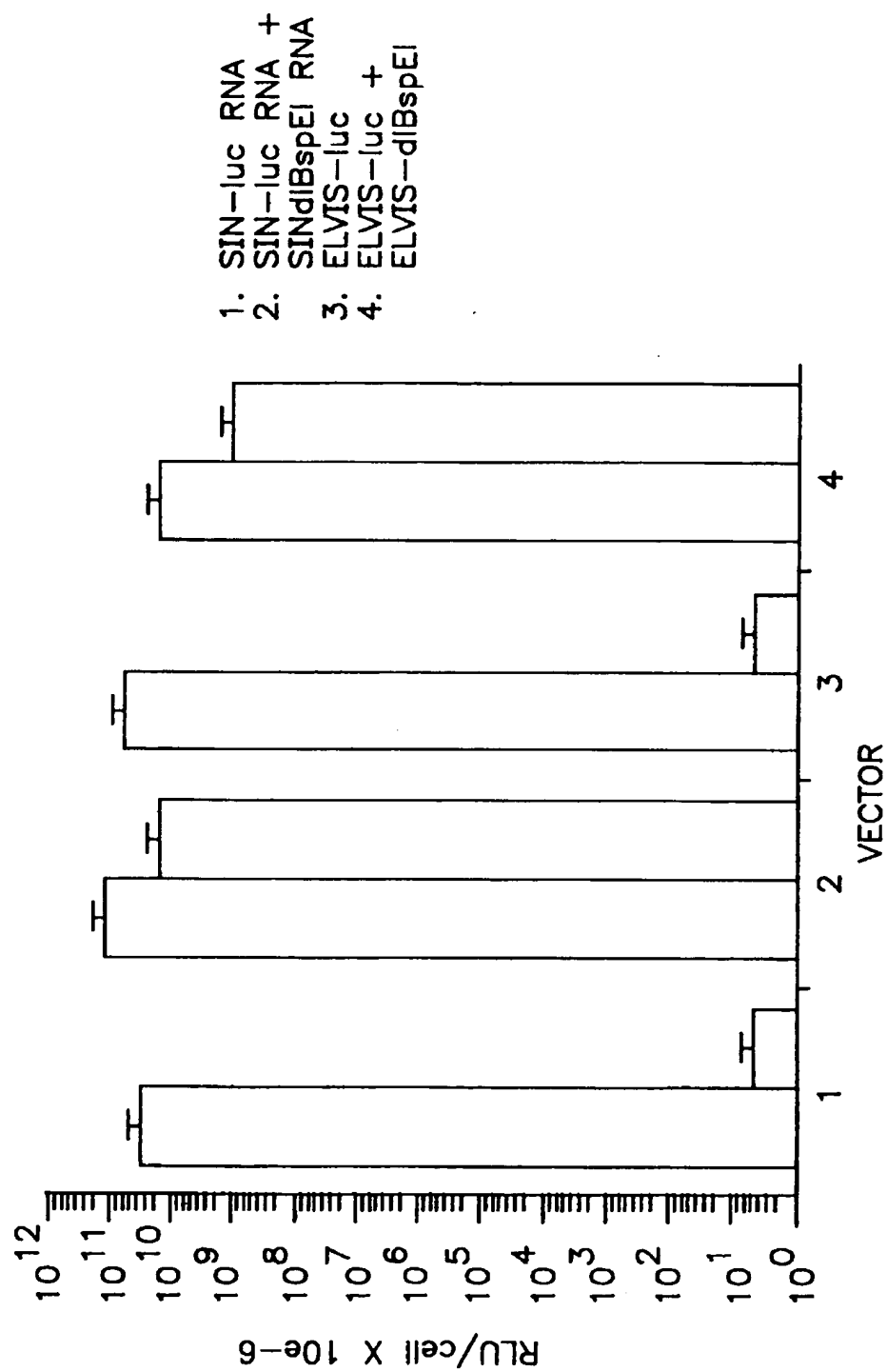
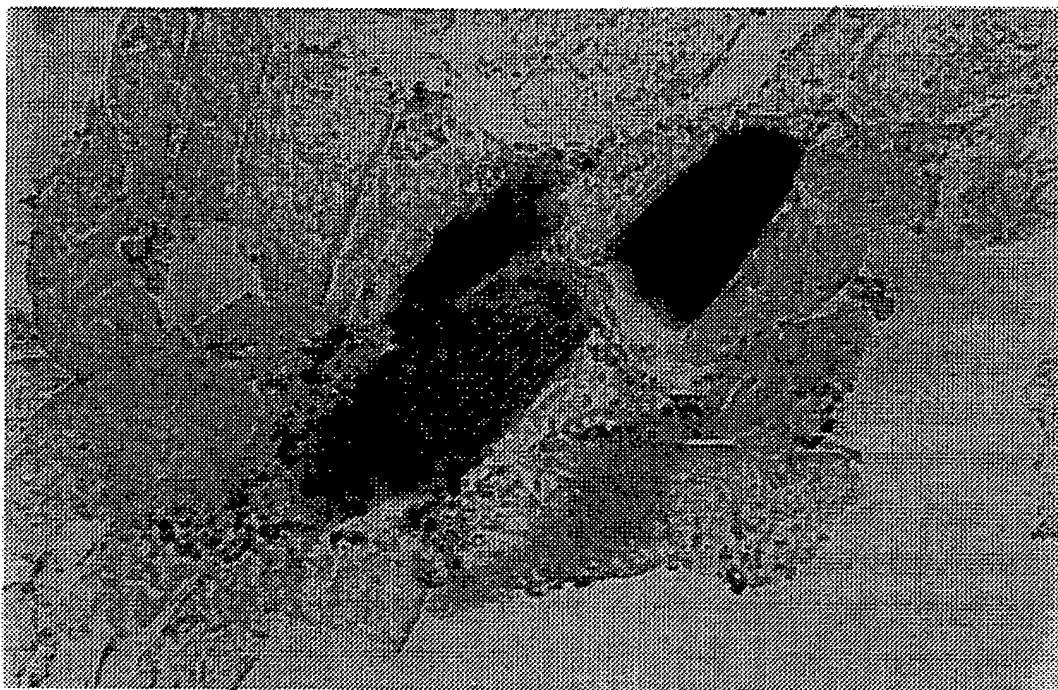


FIG. 23



**FIG. 24**

# EUKARYOTIC LAYERED VECTOR INITIATION SYSTEMS

## CROSS REFERENCE TO RELATED APPLICATIONS

This application is a division of copending U.S. patent application Ser. No. 08/404,796, filed Mar. 15, 1995; which application claims the benefit of PCT application US94/10469, filed Sep. 14, 1994, and is a continuation in part of U.S. patent application Ser. No. 08/376,184, filed Jan. 18, 1995, now abandoned; which application is a continuation-in-part of U.S. patent application Ser. No. 08/348,472, filed Nov. 30, 1994, now abandoned; which application is a continuation-in-part of U.S. patent application Ser. No. 08/198,450, filed Feb. 18, 1994, now abandoned; which application is a continuation-in-part of U.S. patent application Ser. No. 08/122,791, filed Sep. 15, 1993, now abandoned.

## TECHNICAL FIELD

The present invention relates generally to use of recombinant viruses as vectors, and more specifically, to recombinant alphaviruses which are capable of expressing a heterologous sequence in target cells.

## BACKGROUND OF THE INVENTION

Alphaviruses comprise a set of serologically related arthropod-borne viruses of the Togavirus family. Briefly, alphaviruses are distributed worldwide, and persist in nature through a mosquito to vertebrate cycle. Birds, rodents, horses, primates, and humans are among the defined alphavirus vertebrate reservoir/hosts.

Twenty-six known viruses and virus subtypes have been classified within the alphavirus genus utilizing the hemagglutination inhibition (HI) assay. Briefly, the HI test segregates the 26 alphaviruses into three major complexes: the Venezuelan encephalitis (VE) complex, the Semliki Forest (SF) complex, and the western encephalitis (WE) complex. In addition, four additional viruses, eastern encephalitis (EE), Barmah Forest, Middelburg, and Ndumu, receive individual classification based on the HI serological assay.

Members of the alphavirus genus are also classified based on their relative clinical features in humans: alphaviruses associated primarily with encephalitis, and alphaviruses associated primarily with fever, rash, and polyarthritides. Included in the former group are the VE and WE complexes, and EE. In general, infection with this group can result in permanent sequelae, including behavior changes and learning disabilities, or death. In the latter group is the SF complex, comprised of the individual alphaviruses Chikungunya, O'nyong-nyong, Sindbis, Ross River, and Mayaro. With respect to this group, although serious epidemics have been reported, infection is in general self-limiting, without permanent sequelae.

Sindbis virus is the prototype member of the alphavirus genus of the Togavirus family. Although not usually apparent, clinical manifestations of Sindbis virus infection may include fever, arthritis, and rash. Sindbis virus is distributed over Europe, Africa, Asia, and Australia, with the best epidemiological data coming from South Africa, where 20% of the population is seropositive. (For a review, see Peters and Dalrymple, *Fields Virology* (2d ed), Fields et al. (eds.), B.N. Raven Press, New York, N.Y., chapter 26, pp. 713-762). Infectious Sindbis virus has been isolated from human serum only during an outbreak in Uganda and in a single case from Central Africa.

The morphology and morphogenesis of the alphavirus genus is generally quite uniform. In particular, the enveloped 60-65 nm particles infect most vertebrate cells, where productive infection is cytopathic. On the other hand, infection of invertebrate cells, for example, those derived from mosquitoes, does not result in any overt cytopathology. Typically, alphaviruses are propagated in BHK-21 or vero cells, where growth is rapid, reaching a maximum yield within 24 hours of infection. Field strains are usually isolated on primary avian embryo, for example chicken fibroblast cultures (CEF).

The genomic RNA (49S RNA) of alphaviruses is unsegmented, of positive polarity, approximately 11-12 kb in length, and contains a 5' cap and a 3' polyadenylate tail. Infectious enveloped virus is produced by assembly of the viral nucleocapsid proteins onto genomic RNA in the cytoplasm, and budding through the cell membrane embedded with viral-encoded glycoproteins. Entry of virus into cells appears to occur by endocytosis through clathrin-coated pits, fusion of the viral membrane with the endosome, release of the nucleocapsid and uncoating of the viral genome. During viral replication, the genomic 49S RNA serves as template for synthesis of a complementary negative strand. The negative strand in turn serves as template for full-length genomic RNA and for an internally initiated positive-strand 26S subgenomic RNA. The non-structural proteins are translated from the genomic RNA. Alphaviral structural proteins are translated from the subgenomic 26S RNA. All viral genes are expressed as polyproteins and processed into individual proteins by proteolytic cleavage post-translation.

The use of recombinant virus vectors (in particular, alphavirus vectors) to treat individuals requires that they be able to be transported and stored for long periods at a desired temperature, such that infectivity and viability of the recombinant virus is retained. Current methods for storing recombinant viruses generally involve storage as liquids and at low temperatures. Such methods present problems in Third World countries, which typically do not have adequate refrigeration capabilities. For example, each year in Africa, millions of children die from infectious diseases such as measles. Vaccines necessary for the prevention of these diseases cannot be distributed to the majority of these countries because refrigeration is not readily accessible.

In addition to storage as liquids and at low temperatures, present viral formulations often contain media components that are not desirable for injection into patients. Consequently, there is a need in the art for a method of preserving purified recombinant viral vector (and in particular, alphavirus vectors) in a lyophilized form at elevated temperatures, and for this form to be suitable for injection into patients.

The present invention discloses recombinant alphavirus vectors which are suitable for use in a variety of applications, including for example, gene therapy, and further provides other related advantages.

## SUMMARY OF THE INVENTION

Briefly stated, the present invention provides alphavirus vector constructs and alphavirus particles, as well as methods of making and utilizing the same. Within one aspect of the present invention, alphavirus vector constructs are provided comprising a 5' promoter which is capable of initiating the synthesis of viral RNA in vitro from cDNA, a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-

structural proteins, a viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, and an alphavirus RNA polymerase recognition sequence. Within other aspects of the present invention, the viral junction region has been modified such that viral transcription of the subgenomic fragment is reduced.

Within yet other aspects of the present invention, alphavirus vector constructs are provided comprising a 5' promoter which is capable of initiating the synthesis of viral RNA in vitro from cDNA, a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-structural proteins, a first viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, a second viral junction region which is active, or which has been modified such that viral transcription of the subgenomic fragment is reduced, and an alphavirus RNA polymerase recognition sequence.

Within still other aspects of the present invention, alphavirus cDNA vector constructs are provided, comprising a 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA, followed by a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-structural proteins, a viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription termination.

Within another aspect of the present invention, alphavirus cDNA vector constructs are provided, comprising a 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA, followed by a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-structural proteins, a viral junction region which is active, or which has been modified such that viral transcription of the subgenomic fragment is reduced, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription termination.

Within another aspect of the present invention, alphavirus cDNA vector constructs are provided, comprising a promoter which is capable of initiating the synthesis of viral RNA from cDNA followed by a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-structural proteins, a first viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, followed by a second viral junction region which is active, or which has been modified such that viral transcription of the subgenomic fragment is reduced, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription termination.

Within other aspects of the present invention, eukaryotic layered vector initiation systems are provided which are capable of expressing a heterologous nucleic acid sequence in a eukaryotic cell transformed or transfected therewith. In particular embodiments, eukaryotic layered vector initiation systems are provided, comprising a promoter which is capable of initiating the 5' synthesis of RNA from cDNA, a vector construct which is capable of autonomous replication in a cell, the vector construct being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription termination.

Within a related aspect, eukaryotic layered vector initiation systems are provided, comprising a DNA promoter

which is capable of initiating the 5' synthesis of RNA from cDNA, a vector construct which is capable of autonomous replication in a cell, the vector construct being capable of expressing a heterologous ribonucleic acid sequence, and a 3' DNA sequence which controls transcription termination.

Within one embodiment, the vector construct within the eukaryotic layered vector initiation systems of the present invention is an alphavirus vector construct. Within other embodiments, the construct is derived from a virus selected from the group consisting of poliovirus, rhinovirus, coxsackieviruses, rubella, yellow fever, HCV, TGEV, IBV, MHV, BCV, parainfluenza virus, mumps virus, measles virus, respiratory syncytial virus, influenza virus, RSV, MoMLV, HIV, HTLV, hepatitis delta virus and Astrovirus. Within yet other embodiments, the promoter which is capable of initiating the 5' synthesis of RNA from cDNA is selected from the group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40 promoter, and the CMV promoter. Within further embodiments, the eukaryotic layered vector initiation systems further comprise a polyadenylation sequence.

In further embodiments of the invention, in any of the above aspects, the vectors (e.g., alphavirus vector construct, alphavirus cDNA vector construct, or eukaryotic layered vector initiation system) may be derived from an alphavirus selected from the group consisting of Aura, Fort Morgan, Venezuelan Equine Encephalitis, Ross River, Semliki Forest, Sindbis, and Mayaro.

In other embodiments, the vectors described above contain a heterologous sequence. Typically, such vectors contain a heterologous nucleotide sequence of greater than 100 bases, generally the heterologous nucleotide sequence is greater than 3 kb, and sometimes greater than 5 kb, or even 8 kb. In various embodiments, the heterologous sequence is a sequence encoding a protein selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, alpha, beta-, or gamma-IFN, G-CSF, and GM-CSF. Within other embodiments of the invention, the heterologous sequence may encode a lymphokine receptor. Representative examples of such receptors include receptors for any of the lymphokines set forth above.

In still other embodiments, the vectors described above include a selected heterologous sequence which may be obtained from a virus selected from the group consisting of influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and CMV. Within one preferred embodiment, the heterologous sequence obtained from HPV encodes a protein selected from the group consisting of E5, E6, E7 and L1. In yet other embodiments, the vectors described above include a selected heterologous sequence encoding an HIV protein selected from the group consisting of HIV gp120 and gag.

The selected heterologous sequences described above also may be an antisense sequence, noncoding sense sequence, or ribozyme sequence. In preferred embodiments, the antisense or noncoding sense sequence is selected from the group consisting of sequences which are complementary to influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and CMV sequences.

In another embodiment, the vectors described above contain no alphavirus structural protein genes. Within other embodiments, the selected heterologous sequence is located downstream from a viral junction region. In the vectors described above having a second viral junction, the selected heterologous sequence may, within certain embodiments, be

located downstream from the second viral junction region. Where the heterologous sequence is located downstream from a viral junction region, the vector construct may further comprise a polylinker located subsequent to the viral junction region. Within preferred embodiments, such polylinkers do not contain a restriction endonuclease recognition sequence present in the wild-type alphavirus sequence.

In yet another embodiment, in the vectors described above the selected heterologous sequence may be located within the nucleotide sequence encoding alphavirus non-structural proteins.

In particular embodiments, the vectors described above include a viral junction region consisting of the nucleotide sequence as shown in FIG. 3, from nucleotide number 7579, to nucleotide number 7597 (SEQ. ID NO. 1). In alternative embodiments, where the vector includes a second viral junction, an E3 adenovirus gene may be located downstream from the second viral junction region. Vectors of the present invention may also further comprise a non-alphavirus (for example retrovirus, coronavirus, hepatitis B virus) packaging sequence located between the first viral junction region and the second viral junction region, or in the nonstructural protein coding region.

In further aspects, the present invention provides an isolated recombinant alphavirus vector which does not contain a functional viral junction region, and which in preferred embodiments produces reduced viral transcription of the subgenomic fragment.

In still a further aspect, the present invention provides an alphavirus structural protein expression cassette, comprising a promoter and one or more alphavirus structural protein genes, the promoter being capable of directing the expression of alphavirus structural proteins. In various embodiments, the expression cassette is capable of expressing alphavirus structural proteins, such as an alphavirus structural protein selected from the group consisting of C, 6K, E3, E2, and E1.

Within other embodiments, the alphavirus structural protein is derived from an alphavirus selected from the group consisting of Aura, Fort Morgan, Venezuelan Equine Encephalitis, Ross River, Semliki Forest, Sindbis and Mayaro viruses.

In yet another aspect, the present invention provides an alphavirus structural protein expression cassette, comprising a promoter, one or more alphavirus structural proteins, and a heterologous ligand sequence, the promoter being capable of directing the expression of the alphavirus structural proteins and the heterologous sequence. In various embodiments, the heterologous ligand sequence is selected from the group consisting of VSVG, HIV gp120, antibody, insulin, and CD4.

In certain embodiments, the expression cassettes described above include a promoter selected from the group consisting of metallothionein, *Drosophila* actin 5C distal, SV40, heat shock protein 65, heat shock protein 70, Py, RSV, BK, JC, MuLV, MMTV, alphavirus junction region, CMV and VA1RNA.

The present invention also provides packaging cell lines and producer cell lines suitable for producing recombinant alphavirus particles. Such packaging or producer cell lines may be either mammalian or non-mammalian (e.g., insect cells, such as mosquito cells). In certain embodiments, the packaging cell lines and producer cell lines contain an integrated alphavirus structural protein expression cassette.

Within one embodiment, packaging cell lines are provided which, upon introduction of a vector construct, pro-

duce alphavirus particles capable of infecting human cells. Within other embodiments, the packaging cell line produces alphavirus particles in response to one or more factors. Within certain embodiments, an alphavirus inhibitory protein is not produced within the packaging cell line.

Within other aspects, retroviral-derived packaging cell lines are provided which are suitable for packaging and production of an alphavirus vector. Within one embodiment, a retroviral-derived producer cell line suitable for packaging and production of an alphavirus vector is provided, comprising an expression cassette which directs the expression of gag/pol, an expression cassette which directs the expression of env, and alphavirus vector construct containing a retroviral packaging sequence.

Within another aspect, HBV-derived and coronavirus-derived packaging cell lines are provided which are suitable for packaging and production of and alphavirus vector. Within one embodiment, an HBV-derived packaging cell line is provided, comprising an expression cassette which directs the expression of HBV core, preS/S, and P proteins. Within another embodiment, a coronavirus-derived packaging cell line is provided, comprising an expression cassette which directs the expression of coronavirus N, M, and S proteins.

Within another aspect, a VSV-G derived packaging cell is provided which is suitable for packaging and production of an alphavirus vector, comprising a stably integrated expression cassette which directs the expression of VSV-G. Within a further embodiment, such packaging cell lines comprise a stably integrated expression cassette which directs the expression of one or more alphavirus structural proteins.

Within yet other aspects, producer cell lines are provided based upon the above packaging cell lines. Within one embodiment, such producer cell lines produce recombinant alphavirus particles in response to a differentiation state of the producer cell line. Within other embodiments, such producer cell lines produce recombinant alphavirus particles in response to one or more factors.

As utilized with the context of the present invention, alphavirus producer cell line refers to a cell line which is capable of producing recombinant alphavirus particles. The producer cell line should include an integrated alphavirus structural protein expression cassette capable of directing the expression of alphavirus structural protein(s), and also, an alphavirus vector construct. Preferably, the alphavirus vector construct is a cDNA vector construct. More preferably, the alphavirus vector construct is an integrated cDNA vector construct. When the alphavirus vector construct is an integrated cDNA vector construct, it may, in some instances, function only in response to one or more factors, or the differentiation state of the alphavirus producer cell line.

In still yet another aspect, the present invention provides alphavirus particles which, upon introduction into a BHK cell, produces an infected cell which is viable at least 24 hours and as much as 48, 72, or 96 hours, or 1 week after infection. Also provided are mammalian cells which contain such alphavirus particles. In addition, recombinant alphavirus particles capable of infecting human cells are provided.

In another aspect, the present invention provides recombinant alphavirus particles which, upon introduction into a BHK cell, produces an infected cell which is viable at least 24 hours after infection, the particle also carrying a vector construct which directs the expression of at least one antigen or modified form thereof in target cells infected with the alphavirus particle, the antigen or modified form thereof

being capable of stimulating an immune response within an animal. In various embodiments, the expressed antigen or modified form thereof elicits a cell-mediated immune response, preferably an HLA class I-restricted immune response.

In still another aspect, the present invention provides recombinant alphavirus particles which carry a vector capable of directing the expression of a palliative in cells infected with the alphavirus particle, the palliative being capable of inhibiting a function of a pathogenic agent necessary for pathogenicity. In various embodiments, the pathogenic agent is a virus, fungi, protozoa, or bacteria, and the inhibited function is selected from the group consisting of adsorption, replication, gene expression, assembly, and exit of the pathogenic agent from infected cells. In other embodiments, the pathogenic agent is a cancerous cell, cancer-promoting growth factor, autoimmune disorder, cardiovascular disorders such as restenosis, osteoporosis and male pattern baldness, and the inhibited function is selected from the group consisting of cell viability and cell replication. In further embodiments, the vector directs the expression of a toxic palliative in infected target cells in response to the presence in such cells of an entity associated with the pathogenic agent; preferably the palliative is capable of selectively inhibiting the expression of a pathogenic gene or inhibiting the activity of a protein produced by the pathogenic agent. In still further embodiments, the palliative comprises an inhibiting peptide specific for viral protease, an antisense RNA complementary to RNA sequences necessary for pathogenicity, a sense RNA complementary to RNA sequences necessary for pathogenicity, or a defective structural protein of a pathogenic agent, such protein being capable of inhibiting assembly of the pathogenic agent.

In yet further embodiments, recombinant alphavirus particles described above direct the expression of a palliative, more particularly, direct the expression of a gene product capable of activating an otherwise inactive precursor into an active inhibitor of the pathogenic agent, for example, the herpes thymidine kinase gene product, a tumor suppressor gene, or a protein that activates a compound with little or no cytotoxicity into a toxic product in the presence of a pathogenic agent, thereby effecting localized therapy to the pathogenic agent. Alternatively, the recombinant alphavirus particle directs the expression of a protein that is toxic upon processing or modification by a protein derived from a pathogenic agent, a reporting product on the surface of target cells infected with the alphavirus and containing the pathogenic agent, or an RNA molecule which functions as an antisense or ribozyme specific for a pathogenic RNA molecule required for pathogens. In certain embodiments, in the alphavirus particles described above, the protein is herpes thymidine kinase or CD4.

In yet further aspects, the present invention provides recombinant alphavirus particles which direct the expression of a gene capable of suppressing one or more elements of the immune system in target cells infected with the alphavirus vector, and an alphavirus particle which directs the expression of a blocking element in cells infected with the alphavirus vector, the blocking element being capable of binding to either a receptor or an agent such that the receptor/agent interaction is blocked.

In further aspects, methods are provided for administering any of the above-described recombinant alphavirus particles or vectors, for a prophylactic or therapeutic effect. For example, within one aspect, the present invention provides methods of stimulating an immune response to an antigen, comprising the step of infecting susceptible target cells with

a recombinant alphavirus particle which directs the expression of at least one antigen or modified form thereof in target cells infected with the alphavirus, the antigen or modified form thereof being capable of stimulating an immune response within an animal. In one embodiment, the target cells are infected in vivo, although within other embodiments the target cells are removed, infected ex vivo, and returned to the animal.

In still further aspects of the present invention, methods of stimulating an immune response to a pathogenic antigen are provided, comprising the step of infecting susceptible target cells with a recombinant alphavirus particle which directs the expression of a modified form of a pathogenic antigen in target cells infected with the alphavirus, the modified antigen being capable of stimulating an immune response within an animal but having reduced pathogenicity relative to the pathogenic antigen.

In even further aspects of the present invention, methods of stimulating an immune response to an antigen are provided, comprising infecting susceptible target cells with a recombinant alphavirus particle which directs the expression of a peptide having multiple epitopes, one or more of the epitopes derived from different proteins.

In yet another aspect of the invention, methods of stimulating an immune response within a warm-blooded animal are provided, comprising infecting susceptible target cells associated with a warm-blooded animal with nucleic acid sequences coding for either individual class I or class II MHC protein, or combinations thereof, and infecting the cells with an alphavirus particle which directs the expression of at least one antigen or modified form thereof in target cells infected with the alphavirus particle, the antigen or modified form thereof being capable of stimulating an immune response within the animal.

In another aspect of the present invention, methods of inhibiting a pathogenic agent are provided, comprising infecting susceptible target cells with an alphavirus particle which directs the expression of a palliative in cells infected with the alphavirus particle, the palliative being capable of inhibiting a function of a pathogenic agent necessary for pathogenicity.

As utilized within the context of the present invention, vector or vector constructs which direct the expression of a heterologous sequence of interest in fact refers to the transcribed vector RNA, which directs the expression of the heterologous sequence of interest. In addition, although "animals" are generally referred to, it should be understood that the present invention may be readily applied to a wide variety of animals (both mammalian and non-mammalian), including for example, humans, chimps, macaques, cows, horses, sheep, dogs, birds, cats, fish, rats, and mice. Further, although alphaviruses such as Sindbis may be specifically described herein, it should be understood that a wide variety of other alphaviruses may also be utilized including, for example, Aura, Venezuelan Equine Encephalitis, Fort Morgan, Ross River, Semliki Forest, and Mayaro.

Within other aspects of the present invention, methods are provided for delivering a heterologous nucleic acid sequence to an animal comprising the steps of administering to the warm-blooded animal a eukaryotic layered vector initiation system as described above. Within certain embodiments, the eukaryotic layered vector initiation system may be introduced into the target cells directly as a DNA molecule by physical means, as a complex with various liposome formulations, or as a DNA-ligand complex including the vector molecule (e.g., along with a polycation compound

such as polylysine, a receptor specific ligand, or a psoralen inactivated virus such as Sendai or Adenovirus).

Within yet other aspects of the invention, *ex vivo* cells are infected with any of the above-described recombinant alphaviruses are provided. Within yet other aspects, recombinant alphavirus particles are provided which are resistant to inactivation in serum. As utilized herein, recombinant alphavirus particles are considered to be resistant to inactivation in serum if the ratio of surviving particles to input/starting particles in a complement inactivation assay is greater in a statistically significant manner, preferably at least 5-fold, and as much as 10- to 20-fold, as compared to a reference sample produced in BHK cells. Within further aspects, pharmaceutical compositions are provided comprising any of the above-described vectors, or recombinant alphavirus particles, in combination with a physiologically acceptable carrier or diluent.

In yet another aspect of the invention, the eukaryotic layered vector initiation systems provided enable new methods for large scale recombinant protein expression.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (e.g., plasmids, etc.). These references are incorporated herein by reference in their entirety.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration of Sindbis virus genomic organization.

FIG. 2 is an illustration which depicts a method for amplification of a Sindbis RNA genome by RT-PCR.

FIGS. 3A-H set forth the sequence of a representative Eukaryotic Layered Vector Initiation System derived from Sindbis (SEQ. ID NO. 1).

FIG. 4 is a schematic illustration of a Sindbis Basic Vector and a Sindbis-luciferase Vector.

FIG. 5 is an illustration of Sindbis Helper Vector Construction.

FIG. 6 is a graph which illustrates expression and rescue of a Sindbis-luciferase Vector.

FIG. 7 is an illustration of one method for modifying a Sindbis junction region (SEQ ID NO. 1, positions 7579-7602).

FIG. 8 is a schematic illustration of a representative embodiment of a Eukaryotic Layered Vector Initiation System.

FIG. 9 is a graph which shows a time course for luciferase expression from ELVIS-LUC and SINBV-LUC vectors.

FIG. 10 is a bar graph which depicts the level of ELVIS vector reporter gene expression compared to several different vector constructs.

FIG. 11 is a schematic illustration of Sindbis Packaging Expression Cassettes.

FIG. 12 is a bar graph which shows SIN-luc vector packaging by representative packaging cell lines.

FIG. 13 is a bar graph which shows SIN-luc vector packaging by PCL clone #18 over time.

FIG. 14 is a bar graph which depicts the level of expression by several different luciferase vectors in BHK cells and undifferentiated F9 cells.

FIG. 15 is a schematic illustration of how Astroviruses or other heterologous viruses may be used to express Sindbis structural proteins.

FIG. 16A is a bar graph which shows Sindbis BV-HBc expression and packaging in BHK cells (lysate).

FIG. 16B is a bar graph which shows Sindbis BV-HBc expression and packaging in BHK cells (supernatant).

FIG. 17 is a bar graph which shows Sindbis BV-HB core expression and packaging in BHK cells.

FIG. 18 is a bar graph which shows a comparison of HB core expressed from Sindbis and RETROVECTORS™.

FIG. 19 is a bar graph which shows ELVIS-HBc vector expression in BHK cells.

FIGS. 20A-20D are a schematic illustration of several representative mechanisms for activating a disabled viral junction region by "RNA loop-out."

FIG. 21 is a western blot demonstrating expression of capsid protein after transfection with pMAM/C, selection in HAT media, and induction with dexamethasone.

FIGS. 22A-22B depict a bar graph which demonstrates the level of expression of luciferase in BHK cells transfected with ELVIS-LUC vector, and various modifications thereof.

FIG. 23 is a bar graph which demonstrates the level of luciferase or  $\beta$ -galactosidase expression in BHK cells transfected with ELVIS expression vectors, co-transfected with ELVIS expression and helper vectors, or transduced with packaged ELVIS expression vectors.

FIG. 24 depicts a photomicrograph of a ELVIS- $\beta$ -gal injected rat muscle at three days post inoculation. A transverse cryosection from gastronemius muscle injected with 50  $\mu$ g of ELVIS- $\beta$ -gal contained in PBS is shown. Four blue stained transverse fibers are evident.

#### DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Alphavirus vector construct" refers to an assembly which is capable of directing the expression of a sequence(s) or gene(s) of interest. The vector construct should include a 5' sequence which is capable of initiating transcription of an alphavirus, as well as sequence(s) which, when expressed, code for biologically active alphavirus non-structural proteins (e.g., NSP1, NSP2, NSP3, and NSP4), and an alphavirus RNA polymerase recognition sequence. In addition, the vector construct should include a viral junction region which may, in certain embodiments, be modified in order to prevent, increase, or reduce viral transcription of the sub-genomic fragment, and an alphavirus RNA polymerase recognition sequence. The vector may also include nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, a 5' promoter which is capable of initiating the synthesis of viral RNA *in vitro* from cDNA, as well as one or more restriction sites, and a polyadenylation sequence.

"Alphavirus cDNA vector construct" refers to an assembly which is capable of directing the expression of a sequence(s) or gene(s) of interest. The vector construct should include a 5' sequence which is capable of initiating transcription of an alphavirus, as well as sequence(s) which, when expressed, code for biologically active alphavirus non-structural proteins (e.g., NSP1, NSP2, NSP3, and NSP4), and an alphavirus RNA polymerase recognition sequence. In addition, the vector construct should include a 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA, a viral junction region which may, in certain embodiments, be modified in order to prevent,

increase, or reduce viral transcription of the subgenomic fragment, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription termination. The vector may also include nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, splice recognition sequences, a catalytic ribozyme processing sequence, as well as a polyadenylation sequence.

"Expression cassette" refers to a recombinantly produced nucleic acid molecule which is capable of directing the expression of one or more proteins. The expression cassette must include a promoter capable of directing the expression of said proteins, and a sequence encoding one or more proteins, said proteins preferably comprising alphavirus structural protein(s). Optionally, the expression cassette may include transcription termination, splice recognition, and polyadenylation addition sites. Preferred promoters include the CMV, MMTV, MoMLV, and adenovirus VAIRNA promoters. In addition, the expression cassette may contain selectable markers such as Neo, SV2 Neo, hygromycin, phleomycin, histidinol, and DHFR.

"Alphavirus producer cell line" refers to a cell line which is capable of producing recombinant alphavirus particles. The producer cell line should include an integrated alphavirus structural protein expression cassette capable of directing the expression of alphavirus structural protein(s), and also, an alphavirus vector construct. Preferably, the alphavirus vector construct is a cDNA vector construct. More preferably, the alphavirus vector construct is an integrated cDNA vector construct. When the alphavirus vector construct is an integrated cDNA vector construct, it may, in some instances, function only in response to one or more factors, or the differentiation state of the alphavirus producer cell line.

"Recombinant alphavirus particle" refers to a capsid which contains an alphavirus vector construct. Preferably, the capsid is an alphavirus capsid and is contained within a lipid bilayer, such as a cell membrane, in which viral-encoded proteins are embedded. In some instances, the alphavirus vector construct may be contained in a capsid derived from viruses other than alphaviruses (for example, retroviruses, coronaviruses, and hepatitis B virus). A variety of alphavirus vectors may be contained within the recombinant alphavirus particle, including the alphavirus vector constructs of the present invention.

#### A. SOURCES OF ALPHAVIRUS

As noted above, the present invention provides alphavirus vector constructs, alphavirus particles containing such constructs, as well as methods for utilizing such vector constructs and particles. Briefly, sequences encoding wild-type alphavirus suitable for use in preparing the above-described vector constructs and particles may be readily obtained given the disclosure provided herein from naturally-occurring sources, or from depositories (e.g., the American Type Culture Collection, Rockville, Md.).

Representative examples of suitable alphaviruses include Aura (ATCC VR-368), Bebaru virus (ATCC VR-600, ATCC VR-1240), Cabassou (ATCC VR-922), Chikungunya virus (ATCC VR-64, ATCC VR-1241), Eastern equine encephalomyelitis virus (ATCC VR-65, ATCC VR-1242), Fort Morgan (ATCC VR-924), Getah virus (ATCC VR-369, ATCC VR-1243), Kyzylagach (ATCC VR-927), Mayaro (ATCC VR-66), Mayaro virus (ATCC VR-1277), Middleburg (ATCC VR-370), Mucambo virus (ATCC VR-580, ATCC VR-1244), Ndumu (ATCC VR-371), Pixuna virus (ATCC VR-372, ATCC VR-1245), Ross River virus (ATCC VR-373, ATCC VR-1246), Semliki Forest (ATCC VR-67,

ATCC VR-1247), Sindbis virus (ATCC VR-68, ATCC VR-1248), Tonate (ATCC VR-925), Trinita (ATCC VR-469), Una (ATCC VR-374), Venezuelan equine encephalomyelitis (ATCC VR-69), Venezuelan equine encephalomyelitis virus (ATCC VR-923, ATCC VR-1250 ATCC VR-1249, ATCC VR-532), Western equine encephalomyelitis (ATCC VR-70, ATCC VR-1251, ATCC VR-622, ATCC VR-1252), Whataroa (ATCC VR-926), and Y-62-33 (ATCC VR-375).

#### B. SEQUENCES WHICH ENCODE WILD-TYPE SINDBIS VIRUS

Within one particularly preferred aspect of the present invention, the sequences which encode wild-type alphavirus may be obtained from Sindbis virus. In particular, within one embodiment of the invention (and as described in more detail below in Example 1), a Sindbis full-length genomic cDNA clone may be obtained by linking the 5' end of a Sindbis virus cDNA clone to a bacteriophage RNA polymerase promoter, and the 3' end of the cDNA clone to a polyadenosine (poly A) tract of at least 25 nucleotides. In particular, synthesis of the first cDNA strand from the viral RNA template may be accomplished with a 3' oligonucleotide primer having a consecutive sequence comprising an enzyme recognition sequence, a sequence of 25 deoxythymidine nucleotides, and a stretch of approximately 18 nucleotides which is complementary to the viral 3' end, and with a 5' primer containing buffer nucleotides, an enzyme recognition sequence, a bacteriophage promoter, and a sequence complementary to the viral 5' end. The enzyme recognition sites present on each of these primers should be different from each other, and not found in the Sindbis virus. Further, the first nucleotide linked to the 3' end of the bacteriophage RNA polymerase promoter may be the authentic first nucleotide of the RNA virus, or may contain one or more additional non-viral nucleotides. RNA transcribed in vitro from the viral cDNA clone, having the construction described above and linearized by digestion with the unique dT: dA 3' distal restriction enzyme will, after introduction into the appropriate eukaryotic cell, initiate the same infection cycle which is characteristic of infection by the wild-type virus from which the cDNA was cloned. This viral cDNA clone, which yields RNA able to initiate infection after in vitro transcription, is referred to below as an "infectious cDNA clone."

#### C. PRODUCTION OF RECOMBINANT ALPHAVIRUS VECTOR CONSTRUCTS WITH INACTIVATED VIRAL JUNCTION REGIONS

An infectious cDNA clone prepared as described above (or utilizing sequences encoding an alphavirus obtained from other sources) may be readily utilized to prepare alphavirus vector constructs of the present invention. Briefly, within one aspect of the present invention, recombinant alphavirus vector constructs are provided, comprising a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus nonstructural proteins, a viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, and an alphavirus RNA polymerase recognition sequence. As will be discussed in greater detail below, alphavirus vector constructs which have inactivated viral junction regions do not transcribe the subgenomic fragment, making them suitable for a wide variety of applications.

##### 1. RNA POLYMERASE PROMOTER

As noted above, within certain embodiments of the invention alphavirus vector constructs are provided which contain a 5' promoter which is capable of initiating the synthesis of



viral RNA in vitro from cDNA. Particularly, preferred 5' promoters include both eukaryotic and prokaryotic promoters, such as, for example, the  $\beta$ -galactosidase promoter, trpE promoter, lacZ promoter, T7 promoter, T3 promoter, SP6 promoter, SV40 promoter, CMV promoter, and MoMLV LTR.

## 2. SEQUENCES WHICH INITIATE TRANSCRIPTION

As noted above, within preferred embodiments the alphavirus vector constructs of the present invention contain a 5' sequence which is capable of initiating transcription of an alphavirus. Representative examples of such sequences include nucleotides 1-60, and to a lesser extent nucleotides 150-210, of the wild-type Sindbis virus (see FIG. 3), nucleotides 10-75 for tRNA Asparagine (Schlesinger et al., U.S. Pat. No. 5,091,309), and 5' sequences from other Togaviruses which initiate transcription.

## 3. ALPHAVIRUS NONSTRUCTURAL PROTEINS

Alphavirus vector constructs of the present invention should also contain sequences which encode alphavirus nonstructural proteins (NSPs). As an example, for Sindbis virus there are four nonstructural proteins, NSP1, NSP2, NSP3 and NSP4, which encode proteins that enable the virus to self-replicate. Nonstructural proteins 1 through 3 (NSP1-NSP3) are, within one embodiment of the invention, encoded by nucleotides 60 to 5750 of the wild-type Sindbis virus (see FIG. 3). These proteins are produced as a polypeptide and later cleaved into nonstructural proteins NSP1, NSP2, and NSP3. NSP4 is, within one embodiment, encoded by nucleotides 5928 to 7579 (see FIG. 3).

It will be evident to one of ordinary skill in the art that a wide variety of sequences which encode alphavirus nonstructural proteins, in addition to those discussed above, may be utilized in the present invention, and are therefore deemed to fall within the scope of the phrase "Alphavirus Nonstructural Proteins." For example, within one embodiment of the invention, due to the degeneracy of the genetic code, more than one codon may code for a given amino acid. Therefore, a wide variety of nucleic acid sequences which encode alphavirus nonstructural proteins may be generated. Within other embodiments of the invention, a variety of other nonstructural protein derivatives may be made, including for example, various substitutions, insertions, or deletions, the net result of which do not alter the biological activity of the alphavirus nonstructural proteins. Within the context of the present invention, alphavirus nonstructural proteins are deemed to be "biologically active" in toto if they promote the self-replication of the vector construct. Self-replication, which refers to replication of viral nucleic acids and not the production of infectious virus, may be readily determined by metabolic labelling or RNase protection assays performed over a course of time. Methods for making such derivatives may be readily accomplished by one of ordinary skill in the art given the disclosure provided herein (see also, *Molecular Cloning: A Laboratory Manual* (2d. ed.), Cold Spring Harbor Laboratory Press).

## 4. VIRAL JUNCTION REGIONS

Within this aspect of the invention, the alphavirus vector constructs may also include a viral junction region which has been inactivated, such that viral transcription of the subgenomic fragment is prevented. Briefly, the alphavirus viral junction region normally controls transcription initiation of the subgenomic mRNA. In the case of the Sindbis virus, the normal viral junction region typically begins at approximately nucleotide number 7579 and continues through at least nucleotide number 7612 (and possibly beyond). At a minimum, nucleotides 7579 to 7602 (5'-ATC TCT ACG GTG GTC CTAAAT AGT -SEQ. ID NO. 2) are

believed necessary for transcription of the subgenomic fragment. This region (nucleotides 7579 to 7602) is hereinafter referred to as the "minimal junction region core."

Within preferred embodiments of the invention (and as described in more detail below), the viral junction region is inactivated in order to prevent viral transcription of the subgenomic fragment. As utilized within the context of the present invention, "inactivated" means that the fragment corresponding to the initiation point of the subgenomic fragment, as measured by a RNase protection assay, is not detected. (Representative assays are described by Melton et al., *Nuc. Acids Res.* 12:7035-7056, 1984; Calzon et al., *Methods in Enz.* 152:611-632, 1987; and Kekule et al., *Nature* 343:457-461, 1990.)

Within one embodiment of the invention, the viral junction region is inactivated by truncating the viral junction region at nucleotide 7597 (i.e., the viral junction region will then consist of the sequence as shown in FIG. 3, from nucleotide 7579 to nucleotide 7597). This truncation prevents transcription of the subgenomic fragment, and additionally permits synthesis of the complete NSP4 region (which is encoded by nucleotides 5928 to 7579).

As will be evident to one of ordinary skill in the art given the disclosure provided herein, a wide variety of other deletions, substitutions or insertions may also be made in order to inactivate the viral junction region. For example, within other embodiments of the invention the viral junction region may be further truncated into the region which encodes NSP4, thereby preventing viral transcription from the subgenomic fragment while retaining the biological activity of NSP4. Alternatively, within other embodiments, due to the redundancy of the genetic code, nucleotide substitutions may be made in the sequence encoding NSP4, the net effect of which does not alter the biological activity of NSP4 yet, nevertheless, prevents transcription of the subgenomic fragment.

## 5. ALPHAVIRUS RNA POLYMERASE RECOGNITION SEQUENC. AND POLY-A TAIL

As noted above, alphavirus vector constructs of the present invention should also include an alphavirus RNA polymerase recognition sequence (also termed "alphavirus replicase recognition sequence"). Briefly, the alphavirus RNA polymerase recognition sequence provides a recognition site at which the virus begins replication by synthesis of the negative strand. A wide variety of sequences may be utilized as an alphavirus RNA polymerase recognition sequence. For example, within one embodiment, Sindbis vector constructs of the present invention include a Sindbis polymerase recognition sequence which is encoded by nucleotides 11,647 to 11,703 (see FIG. 3). Within other embodiments, the Sindbis polymerase recognition is truncated to the smallest region which can still function as a recognition sequence (e.g., nucleotides 11,684 to 11,703 of FIG. 3).

Within preferred embodiments of the invention, the vector construct may additionally contain a polyA tail. Briefly, the polyA tail may be of any size which is sufficient to promote stability in the cytoplasm, thereby increasing the efficiency of initiating the viral life cycle. Within various embodiments of the invention, the polyA tail comprises at least 10 adenosine nucleotides, and most preferably, at least 25 adenosine nucleotides.

## D. OTHER ALPHAVIRUS VECTOR CONSTRUCTS

In addition to the vector constructs which are generally described above, a wide variety of other alphavirus vector constructs may also be prepared utilizing the disclosure provided herein.

### 1. MODIFIED VIRAL JUNCTION REGIONS

As noted above, the present invention provides viral junction regions which have been modified from the wild-type sequence. Within the context of the present invention, modified viral junction regions should be understood to include junction regions which have wild-type activity, but a non-wild-type sequence, as well as junction regions with increased, decreased, or no activity. For example, within one aspect of the invention, alphavirus vector constructs are provided wherein the viral junction region has been modified, such that viral transcription of the subgenomic fragment is reduced. Briefly, infection of cells with wild-type alphavirus normally results in cell death as a result of abundant viral transcription of the subgenomic fragment initiated from the viral junction region. This large abundance of RNA molecules can overwhelm the transcriptional machinery of the infected cell, ultimately resulting in death of the cell. In applications where it is desired that infection of a target cell should result in a therapeutic effect (e.g., strand scission of a target nucleic acid or prolonged expression of a heterologous protein) rather than cell death, several modifications to the alphavirus vector construct (in addition to inactivating the vector construct, as described above) may be made in order to reduce the level of viral transcription of the subgenomic fragment, and thereby prolong the life of the vector infected target cell. Within the context of the present invention, viral transcription of the subgenomic fragment is considered to be "reduced" if it produces less subgenomic fragment than a standard wild-type alphavirus (e.g., Sindbis virus ATCC No. VR-1248) as determined by a RNase protection assay.

Viral junction regions may be modified by a variety of methods in order to reduce the level of viral transcription of the subgenomic fragment. For example, within one embodiment of the invention, due to the redundancy of the genetic code nucleotide substitutions may be made in the viral junction region 7579 to 7597, the net effect of which does not alter the amino acid sequence NSP4 (or, within other embodiments, the biological activity of NSP4), and yet reduces the level of viral transcription of the subgenomic fragment. If the modified vector construct includes nucleotides beyond 7597 (e.g., to 7602 or 7612), further nucleotide substitutions may likewise be made, although, since NSP4 terminates at 7597, such substitutions need not be based upon genetic redundancy. Representative examples of modified viral junction regions are described in more detail below in Example 3.

### 2. TANDEM VIRAL JUNCTION REGIONS

Within other aspects of the invention, alphavirus vector constructs are provided, which comprise a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-structural proteins, a first viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, a second viral junction region which is active, or which has been modified such that viral transcription of the subgenomic fragment is reduced, and an alphavirus RNA polymerase recognition sequence. Such vector constructs are referred to as "tandem" vector constructs because they comprise a first inactivated (or "disabled") viral junction region, as well as a second modified ("synthetic") or unmodified viral junction region. Within preferred embodiments of the invention, the inactivated junction region is followed directly by the second viral junction region.

In applications where a low level of subgenomic transcription is required, a minimal junction region core may be

inserted downstream in tandem to the inactivated junction region. In order to gradually increase the level of subgenomic transcription for the desired effect, sequences corresponding to the entire junction region may be added to the in-tandem junction region, in increments.

### 3. THE ADENOVIRUS E3 GENE

Within another aspect of the invention, an adenovirus E3 gene is inserted into a tandem vector construct following the second viral junction region, in order to down-regulate HLA expression in alphavirus infected cells. Briefly, within various embodiments of the invention, repeated inoculations of a gene therapeutic into the same individual is desirable. However, repeated inoculations of alphaviruses such as the Sindbis virus may lead to the development of specific antibodies or cell-mediated immune response against Sindbis viral nonstructural proteins (NSPs). Thus, it may be necessary to mitigate the host immune response targeted to vector-specific proteins in order to administer repeated doses to the same individual.

Therefore, within one embodiment of the invention, products of the Adenovirus type 2 early region gene 3 are utilized in order to down-regulate the expression of integral histocompatibility antigens expressed on the surface of infected cells. Briefly, the E3 19,000 dalton (E3/19K) protein binds to, and forms a molecular complex with, class I H-2/HLA antigens in the endoplasmic reticulum, preventing terminal glycosylation pathways necessary for the full maturation and subsequent transport of the class I H-2/HLA antigens to the cell membrane. In target cells infected with an alphavirus vector encoding the Ad 2 E3 protein, co-expression of the viral nonstructural proteins in the context of class I antigens will not occur. Thus, it is possible to administer repeated doses of an alphavirus vector which expresses the Ad 2 E3 protein as a component of its therapeutic palliative to the same individual. A representative example of the use of the Adenovirus E3 gene is set forth in more detail below in Example 4A.

### 4. THE CMV H301 GENE

Other methods may also be utilized in order to mitigate a host's immune response against viral NSPs. For example, within another aspect of the invention, the human cytomegalovirus ("HCMV") H301 gene is cloned into an alphavirus vector construct, preferably immediately following the second viral junction region in a tandem vector, in order to inhibit host CTL response directed against viral specific proteins expressed in vector infected cells.

Briefly, 2-Microglobulin (2m) protein binds to the 1, 2 and 3 domains of the alpha-chain of the class I major histocompatibility molecules of higher eukaryotes. Preventing the interaction between 2m and MHC class I products renders infected cells unrecognizable by cytotoxic T cells. Therefore, as described in greater detail below in Example 4B, expression of the HCMV H301 gene product as a component of a therapeutic palliative may be utilized in order to mitigate the host immune response to viral NSP.

### 5. NONALPHAVIRUS PACKAGING SEQUENCE

Within another aspect of the invention, a packaging sequence derived from a virus other than an alphavirus (for example, retrovirus, coronavirus, hepatitis B virus) is inserted into a tandem vector and positioned between the first (inactivated) viral junction region and the second, modified viral junction region. Briefly, nonalphavirus packaging sequences signal the packaging of an RNA genome into a virus particle corresponding to the source of the packaging sequence. For example, and as described in more detail below, a retroviral packaging sequence may be utilized in order to package an alphavirus vector into a retro-

viral particle using a retroviral packaging cell line. This is performed in order to increase the efficiency of alphavirus vector transfer into an alphavirus packaging cell line, or to alter the cell or tissue tropism of the alphavirus vector.

## 6. EXPRESSION OF MULTIPLE HETEROLOGOUS GENES

The genomic length and subgenomic length of mRNAs transcribed in wild-type alphavirus infected cells are polycistronic, coding for, respectively, the viral four non-structural proteins (NSPs) and four structural proteins (SPs). The genomic and subgenomic mRNAs are translated as polyproteins, and processing into the individual nonstructural and structural proteins is accomplished by post-translational proteolytic cleavage, catalyzed by viral encoded NSP- and SP- specific proteases, as well as cellular proteases.

In certain applications of the alphavirus vectors described herein, the expression of more than one heterologous gene is desired. For example, in order to treat metabolic disorders such as Gaucher's syndrome, multiple administrations of alphavirus vectors or particles may be required, since duration of the therapeutic palliative may be limited. Therefore, with certain embodiments of the invention it may be desirable to co-express in a target cell the Ad 2 E3 gene (see Example 4), along with a therapeutic palliative, such as the glucocerebrosidase gene (see Example 17). In wild-type virus, however, the structural protein ("SP") polycistronic message is translated into a single polyprotein which is subsequently processed into individual proteins by cleavage with SP-encoded proteases. Thus, expression of multiple heterologous genes from a polycistronic message requires a mechanism different from the wild-type virus, since the SP protease gene, or the peptides recognized for cleavage, are not present in the replacement region of the alphavirus vectors.

Therefore, within one embodiment of the invention alphavirus vectors may be constructed by placing appropriate signals either ribosome readthrough or internal ribosome entry between cistrons. One such representative method of expressing multiple heterologous genes is set forth below in Example 5.

In yet another embodiment of the invention, the placement of signals promoting either ribosome readthrough or internal ribosome entry immediately downstream of the disabled junction region vector pKSSINBVd1JR is described (see Examples 3 and 5). In this vector configuration, synthesis of subgenomic message cannot occur; however, the heterologous proteins are expressed from genomic length mRNA by either ribosomal readthrough (scanning) or internal ribosome entry. Relative to wild-type, the low level of viral transcription with this alphavirus vector would prolong the life of the infected target cell.

In still another embodiment of the invention, placement of signals promoting either ribosome readthrough or internal ribosome entry immediately downstream of the pKSSINBVd1JRsjr or pKSSINBV vectors is described. Briefly, since synthesis of subgenomic mRNA occurs in cells infected with the pKSSINBVd1JRsjr and pKSSINBV vectors, placement of either a ribosome readthrough sequence or an internal ribosome entry sequence between the two heterologous genes permits translation of both proteins encoded by the subgenomic mRNA polycistronic message. Further, additional heterologous genes can be placed in the subgenomic mRNA region, provided that a suitable translation initiation signal resides at the 5' end of the translational AUG start codon. The number of heterolo-

gous gene(s) which can be inserted into the subgenomic mRNA region, as described here, is limited only by the packaging constraints of the vector.

Different sequences which allow either ribosome readthrough, cap-independent translation, or internal ribosome entry may be placed into Sindbis vectors pKSSINBVd1JR, pKSSINBV, pKSSINBVd1JRsjr, or vectors encompassed by the eukaryotic layered vector initiation system, in the configurations as discussed above. The source of these translation control sequences are the picornaviruses polio and EMCV, the 5' noncoding region of the human immunoglobulin heavy-chain binding protein, and a synthetic sequence of at least 15 bps corresponding in part to the Kozak consensus sequence for efficient translational initiation. Although not described in detail here, these signals which affect translation initiation can also be placed downstream of the junction region and between heterologous genes in all of the modified junction region vectors described in Example 3.

As noted above, the alphavirus cDNA vector construct also includes a 3' sequence which controls transcription termination. A representative example of such a sequence is set forth in more detail below in Examples 2 and 3.

## 7. TISSUE SPECIFIC EXPRESSION

Within other aspects of the present invention, alphavirus vector constructs are provided which are capable of expressing a desired heterologous sequence only in a selected tissue. One such representative example is shown in FIG. 20. Briefly, as shown in FIG. 20A, a recombinant alphavirus vector is constructed such that upon introduction of the vector (FIG. 20A) into a target cell, internal inverted repeat sequences which flank the transcriptional control regions (e.g., modified junction region) loop out (see FIG. 20B), thereby preventing viral transcription of subgenomic sequences ("G.O.I.") from the synthetic junction region.

On the other hand, activation of the vector can be attained if the inverted repeats are designed to also hybridize to a specific cellular RNA sequence which is characteristic of a selected tissue or cell type. Such cellular RNA disrupts the disabling stem loop structure, thereby allowing the formation of a more stable secondary stem loop structure (FIGS. 20C and 20D). This secondary stem loop structure allows transcription of the sub-genomic message by placing the junction region back into its correct positional configuration.

Full-length alphavirus vectors can also be transcribed using the secondary stem loop structure by taking advantage of the ability of the viral polymerase to switch templates during synthesis of the negative strand using a strand hopping mechanism termed copy choice (King, *RNA genetics II*, CRC Press, Inc., Boca Raton Fla., Domingo et al. (ed.), pp. 150-185, 1988). Once a single successful round of transcription has occurred, the resulting RNA transcript does not contain inverted repeats because they are deleted as a result of the polymerase copy choice event. This newly synthesized RNA molecule now functions as the primary RNA vector transcript which will transcribe and express as any other non-disabled genomic alphavirus vector previously described. In this RNA vector configuration, tissue or cell-specific activation of the disabled Sindbis vector can be achieved if specific RNA sequences, present only in the targeted cell or tissue types, are used in the design of the inverted repeats. In this fashion alphaviruses such as Sindbis can be engineered to be tissue-specific expression vectors using similar inverted sequences described above.

Using this vector system to achieve tissue specific expression enables a therapeutic alphavirus vector or particle to be delivered systemically into a patient. If the vector should

infect a cell which does not express the appropriate RNA species, the vector will only be capable of expressing nonstructural proteins and not the gene of interest. Eventually, the vector will be harmlessly degraded.

Use of the above-described vectors enables virtual tissue-specific expression possible for a variety of therapeutic applications, including for example, targeting vectors for the treatment for various types of cancers. This rationale relies on specific expression of tumor-specific markers such as the carcinoembryonic tumor specific antigen (CEA) and the alpha-fetoprotein tumor marker. Briefly, utilizing such tumor-specific RNA to target specific tumors allows for the tumor-specific expression of toxic molecules, lymphokines or pro-drugs discussed below. Such methods may be utilized for a wide variety of tumors, including for example, colorectal, lung, breast, ovary, bladder and prostate cancers because all these tumors express the CEA. One representative illustration of vectors suitable for use within this aspect of the present invention is set forth in more detail below in Example 16.

Briefly, CEA was one of the first tumor-specific markers to be described, along with the alpha-fetoprotein tumor marker. CEA is a normal glycoprotein in the embryonic tissue of the gut, pancreas and liver during the first two trimesters of fetal development (*Pathologic Basis of Disease*, 3rd edition 1984, Robbins et al. (eds.)). Previously, CEA was believed to be specific for adenocarcinomas of the colon, however, with the subsequent development of more sensitive radioimmunoassays it became apparent that CEA was presented in the plasma with many endodermally derived cancers, particularly pancreatic, gastric and bronchogenic.

Within related aspects of the present invention, alphavirus cell-specific expression vectors may be constructed to express viral antigens, ribozyme, antisense sequences or immunostimulatory factors such as gamma-interferon ( $\gamma$ -IFN), IL-2 or IL-5 for the targeted treatment of virus infected cell types. In particular, in order to target alphavirus vectors to specific foreign organism or pathogen-infected cells, inverted repeats of the alphavirus vector may be selected to hybridize to any pathogen-specific RNA, for instance target cells infected by pathogens such as HIV, CMV, HBV, HPV and HSV.

Within yet other aspects of the invention, specific organ tissues may be targeted for the treatment of tissue-specific metabolic diseases utilizing gene replacement therapies. For example, the liver is an important target tissue because it is responsible for many of the body's metabolic functions and is associated with many metabolic genetic disorders. Such diseases include many of the glycogen storage diseases, phenylketonuria, Gaucher's disease and familial hypercholesterolemia. Presently there are many liver-specific enzymes and markers which have been sequenced which may be used to engineer appropriate inverted repeats for alphavirus vectors. Such liver-specific cDNAs include sequences encoding for S-adenosylmethionine synthetase (Horikawa et al., *Biochem. Int.* 25:81, 1991); lecithin: cholesterolacyl transferase (Rogne et al., *Biochem. Biophys. Res. Commun.* 148:161, 1987); as well as other liver-specific cDNAs (Chin et al., *Ann. N.Y. Acad. Sci.* 478:120, 1986). Such a liver-specific alphavirus vector could be used to deliver the low density lipoprotein receptor (Yamamoto et al., *Cell* 39:27, 1984) to liver cells for the treatment of familial hypercholesterolemia (Wilson et al., *Mol. Biol. Med.* 7:223, 1990).

#### E. HETEROLOGOUS SEQUENCES

As noted above, a wide variety of nucleotide sequences may be carried by the alphavirus vector constructs of the

present invention. Preferably, the nucleotide sequences should be of a size sufficient to allow production of viable virus. Within the context of the present invention, the production of any measurable titer, for example, by plaque assay, luciferase assay, or  $\beta$ -galactosidase assay, of infectious virus on appropriate susceptible monolayers, is considered to be "production of viable virus." This may be, at a minimum, an alphavirus vector construct which does not contain any additional heterologous sequence. However, within other embodiments, the vector construct may contain additional heterologous or foreign sequences. Within preferred embodiments, the heterologous sequence will comprise a heterologous sequence of at least about 100 bases, 2 kb, 3.5 kb, 5 kb, 7 kb, or even a heterologous sequence of at least about 8 kb.

As will be evident to one of ordinary skill in the art given the disclosure provided herein, the efficiency of packaging and hence, viral titer, is to some degree dependent upon the size of the sequence to be packaged. Thus, in order to increase the efficiency of packaging and the production of viable virus, additional non-coding sequences may be added to the vector construct. Moreover, within certain embodiments of the invention it may be desired to increase or decrease viral titer. This increase or decrease may be accomplished by increasing or decreasing the size of the heterologous sequence, and hence the efficiency of packaging.

A wide variety of heterologous sequences may be included in the vector construct, including for example sequences which encode palliatives such as lymphokines, toxins, prodrugs, antigens which stimulate an immune response, ribozymes, and proteins which assist or inhibit an immune response, as well as antisense sequences (or sense sequences for "antisense applications"). As noted above, within various embodiments of the invention the alphavirus vector constructs provided herein may contain (and express, within certain embodiments) two or more heterologous sequences.

#### 1. LYMPHOKINES

Within one embodiment of the invention, the heterologous sequence encodes a lymphokine. Briefly, lymphokines act to proliferate, activate, or differentiate immune effectors cells. Representative examples of lymphokines include gamma interferon, tumor necrosis factor, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF, CSF-1 and G-CSF.

Within related embodiments of the invention, the heterologous sequence encodes an immunomodulatory cofactor. Briefly, as utilized within the context of the present invention, "immunomodulatory cofactor" refers to factors which, when manufactured by one or more of the cells involved in an immune response, or when added exogenously to the cells, causes the immune response to be different in quality or potency from that which would have occurred in the absence of the cofactor. The quality or potency of a response may be measured by a variety of assays known to one of skill in the art including, for example, in vitro assays which measure cellular proliferation (e.g.,  $^3\text{H}$  thymidine uptake), and in vitro cytotoxic assays (e.g., which measure  $^{51}\text{Cr}$  release) (see Warner et al., *AIDS Res. and Human Retroviruses* 7:645-655, 1991).

Representative examples of immunomodulatory co-factors include alpha interferon (Finter et al., *Drugs* 42(5):749-765, 1991; U.S. Pat. No. 4,892,743; U.S. Pat. No. 4,966,843; WO 85/02862; Nagata et al., *Nature* 284:316-320, 1980; Familletti et al., *Methods in Enz.* 78:387-394, 1981; Twu et al., *Proc. Natl. Acad. Sci. USA* 86:2046-2050, 1989; Faktor et al., *Oncogene* 5:867-872,

1990), beta interferon (Seif et al., *J. Virol.* 65:664-671, 1991), gamma interferons (Radford et al., *American Society of Hepatology*:2008-2015, 1991; Watanabe et al., *PNAS* 86:9456-9460, 1989; Gansbacher et al., *Cancer Research* 50:7820-7825, 1990; Maio et al., *Can. Immunol. Immunother.* 30:34-42, 1989; U.S. Pat. Nos. 4,762,791 and 4,727,138), G-CSF (U.S. Pat. Nos. 4,999,291 and 4,810,643), GM-CSF (WO 85/04188), TNFs (Jayaraman et al., *J. Immunology* 144:942-951, 1990), Interleukin-2 (IL-2) (Karupiah et al., *J. Immunology* 144:290-298, 1990; Weber et al., *J. Exp. Med.* 166:1716-1733, 1987; Gansbacher et al., *J. Exp. Med.* 172:1217-1224, 1990; U.S. Pat. No. 4,738,927), IL-4 (Tepper et al., *Cell* 57:503-512, 1989; Golumbek et al., *Science* 254:713-716, 1991; U.S. Pat. No. 5,017,691), IL-6 (Brakenhof et al., *J. Immunol.* 139:4116-4121, 1987; WO 90/06370), IL-12, IL-15 (Grabstein et al., *Science* 264:965-968, 1994; Genbank-EMBL Accession No. V03099), ICAM-1 (Altman et al., *Nature* 338:512-514, 1989), ICAM-2, LFA-1, LFA-3, MHC class I molecules, MHC class II molecules,  $\alpha_2$ -microglobulin, chaperones, CD3, B7/BB 1, MHC linked transporter proteins or analogues thereof.

The choice of which immunomodulatory cofactor to include within an alphavirus vector construct may be based upon known therapeutic effects of the cofactor, or experimentally determined. For example, in chronic hepatitis B infections alpha interferon has been found to be efficacious in compensating a patient's immunological deficit and thereby assisting recovery from the disease. Alternatively, a suitable immunomodulatory cofactor may be experimentally determined. Briefly, blood samples are first taken from patients with a hepatic disease. Peripheral blood lymphocytes (PBLs) are restimulated in vitro with autologous or HLA-matched cells (e.g., EBV transformed cells), and transduced with an alphavirus vector construct which directs the expression of an immunogenic portion of a hepatitis antigen and the immunomodulatory cofactor. Stimulated PBLs are used as effectors in a CTL assay with the HLA-matched transduced cells as targets. An increase in CTL response over that seen in the same assay performed using HLA-matched stimulator and target cells transduced with a vector encoding the antigen alone, indicates a useful immunomodulatory cofactor. Within one embodiment of the invention, the immunomodulatory cofactor gamma interferon is particularly preferred.

Another example of an immunomodulatory cofactor is the B7/BB1 costimulatory factor. Briefly, activation of the full functional activity of T cells requires two signals. One signal is provided by interaction of the antigen-specific T cell receptor with peptides which are bound to major histocompatibility complex (MHC) molecules, and the second signal, referred to as costimulation, is delivered to the T cell by antigen-presenting cells. Briefly, the second signal is required for interleukin-2 (IL-2) production by T cells and appears to involve interaction of the B7/BB 1 molecule on antigen-presenting cells with CD28 and CTLA-4 receptors on T lymphocytes (Linsley et al., *J. Exp. Med.*, 173:721-730, 1991a, and *J. Exp. Med.*, 174:561-570, 1991). Within one embodiment of the invention, B7/BB1 may be introduced into tumor cells in order to cause costimulation of CD8<sup>+</sup> T cells, such that the CD8<sup>+</sup> T cells produce enough IL-2 to expand and become fully activated. These CD8<sup>+</sup> T cells can kill tumor cells that are not expressing B7 because costimulation is no longer required for further CTL function. Vectors that express both the costimulatory B7/BB 1 factor and, for example, an immunogenic HBV core protein, may be made utilizing methods which are described herein. Cells

transduced with these vectors will become more effective antigen-presenting cells. The HBV core-specific CTL response will be augmented from the fully activated CD8<sup>+</sup> T cell via the costimulatory ligand B7/BB1.

## 2. TOXINS

Within another embodiment of the invention, the heterologous sequence encodes a toxin. Briefly, toxins act to directly inhibit the growth of a cell. Representative examples of toxins include ricin (Lamb et al., *Eur. J. Biochem.* 148:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem.* 198:723-732, 1991; Evensen et al., *J. of Biol. Chem.* 266:6848-6852, 1991; Collins et al., *J. of Biol. Chem.* 265:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem Soc.* 309:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem.* 260:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature* 306:551-557, 1983; Sanchez and Holmgren, *PNAS* 86:481-485, 1989), gelonin (Stirpe et al., *J. Biol. Chem.* 255:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther.* 21:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J.* 203:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys.* 200:418-425, 1980; Irvin, *Arch. Biochem. & Biophys.* 169:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS* 84:4364-4368, 1987; Jackson et al., *Microb. Path.* 2:147-153, 1987), Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem.* 262:8707-8711, 1987), herpes simplex virus thymidine kinase (HSVTK) (Field et al., *J. Gen. Virol.* 49:115-124, 1980), and *E. coli* guanine phosphoribosyl transferase.

## 3. PRO-DRUGS

Within other embodiments of the invention, the heterologous sequence encodes a "pro-drug". Briefly, as utilized within the context of the present invention, "pro-drug" refers to a gene product that activates a compound with little or no cytotoxicity into a toxic product. Representative examples of such gene products include HSVTK and VZVTK (as well as analogues and derivatives thereof), which selectively monophosphorylate certain purine arabinosides and substituted pyrimidine compounds, converting them to cytotoxic or cytostatic metabolites. More specifically, exposure of the drugs ganciclovir, acyclovir, or any of their analogues (e.g., FIAU, FIAC, DHPG) to HSVTK phosphorylates the drug into its corresponding active nucleotide triphosphate form.

Representative examples of other pro-drugs which may be utilized within the context of the present invention include: *E. coli* guanine phosphoribosyl transferase which converts thioxanthine into toxic thioxanthine monophosphate (Besnard et al., *Mol Cell Biol.* 7:4139-4141, 1987); alkaline phosphatase, which will convert inactive phosphorylated compounds such as mitomycin phosphate and doxorubicin-phosphate to toxic dephosphorylated compounds; fungal (e.g., *Fusarium oxysporum*) or bacterial cytosine deaminase, which will convert 5-fluorocytosine to the toxic compound 5-fluorouracil (Mullen, *PNAS* 89:33, 1992); carboxypeptidase G2, which will cleave the glutamic acid from para-N-bis (2-chloroethyl) aminobenzoyl glutamic acid, thereby creating a toxic benzoic acid mustard; and Penicillin-V amidase, which will convert phenoxycarbide derivatives of doxorubicin and melphalan to toxic compounds (see generally, Vrudhula et al., *J. of Med. Chem.* 36(7):919-923, 1993; Kern et al., *Canc. Immun. Immunother.* 31(4):202-206, 1990).

## 4. ANTISENSE SEQUENCES

Within another embodiment of the invention, the heterologous sequence is an antisense sequence. Briefly, antisense sequences are designed to bind to RNA transcripts, and thereby prevent cellular synthesis of a particular protein or prevent use of that RNA sequence by the cell. Represent-

tative examples of such sequences include antisense thymidine kinase, antisense dihydrofolate reductase (Maher and Dolnick, *Arch. Biochem. & Biophys.* 253:214-220, 1987; Bzik et al., *PNAS* 84:8360-8364, 1987), antisense HER2 (Coussens et al., *Science* 230:1132-1139, 1985), antisense ABL (Fainstein et al., *Oncogene* 4:1477-1481, 1989), antisense Myc (Stanton et al., *Nature* 310:423-425, 1984) and antisense ras, as well as antisense sequences which block any of the enzymes in the nucleotide biosynthetic pathway. In addition, within other embodiments of the invention antisense sequences to interferon and 2 microglobulin may be utilized in order to decrease immune response.

In addition, within a further embodiment of the invention, antisense RNA may be utilized as an anti-tumor agent in order to induce a potent Class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences are believed to induce the increased expression of interferons (including gamma-interferon) due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA. Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

#### 5. RIBOZYMES

Within other aspects of the present invention, alphavirus vectors are provided which produce ribozymes upon infection of a host cell. Briefly, ribozymes are used to cleave specific RNAs and are designed such that it can only affect one specific RNA sequence. Generally, the substrate binding sequence of a ribozyme is between 10 and 20 nucleotides long. The length of this sequence is sufficient to allow a hybridization with target RNA and disassociation of the ribozyme from the cleaved RNA. Representative examples for creating ribozymes include those described in U.S. Pat. Nos. 5,116,742; 5,225,337 and 5,246,921. Particularly preferred ribozymes for use within the present invention include those disclosed in more detail below in the Examples (e.g., Examples 18 and 19).

#### 6. PROTEINS AND OTHER CELLULAR CONSTITUENTS

Within other aspects of the present invention, a wide variety of proteins or other cellular constituents may be carried by the alphavirus vector construct. Representative examples of such proteins include native or altered cellular components, as well as foreign proteins or cellular constituents, found in for example, viruses, bacteria, parasites or fungus.

##### (a) Altered Cellular Components

Within one embodiment, alphavirus vector constructs are provided which direct the expression of an immunogenic, non-tumorigenic, altered cellular component. As utilized herein, the term "immunogenic" refers to altered cellular components which are capable, under the appropriate conditions, of causing an immune response. This response must be cell-mediated, and may also include a humoral response. The term "non-tumorigenic" refers to altered cellular components which will not cause cellular transformation or induce tumor formation in nude mice. The phrase "altered cellular component" refers to proteins and other cellular constituents which are either associated with rendering a cell tumorigenic, or are associated with tumorigenic cells in general, but are not required or essential for rendering the cell tumorigenic.

Before alteration, the cellular components may be essential to normal cell growth and regulation and include, for example, proteins which regulate intracellular protein degradation, transcriptional regulation, cell-cycle control, and cell-cell interaction. After alteration, the cellular com-

ponents no longer perform their regulatory functions and, hence, the cell may experience uncontrolled growth. Representative examples of altered cellular components include ras\*, p53\*, Rb\*, altered protein encoded by the Wilms' tumor gene, ubiquitin\*, mucin\*, protein encoded by the DCC, APC, and MCC genes, the breast cancer gene BRCA1\*, as well as receptors or receptor-like structures such as neu, thyroid hormone receptor, platelet derived growth factor (PDGF) receptor, insulin receptor, epidermal growth factor (EGF) receptor, and the colony stimulating factor (CSF) receptor.

Within one embodiment of the present invention, alphavirus vector constructs are provided which direct the expression of a non-tumorigenic, altered ras (ras\*) gene. Briefly, the ras\* gene is an attractive target because it is causally linked to the neoplastic phenotype, and indeed may be necessary for the induction and maintenance of tumorigenesis in a wide variety of distinct cancers, such as pancreatic carcinoma, colon carcinoma and lung adenocarcinoma. In addition, ras\* genes are found in pre-neoplastic tumors and, therefore, immune intervention therapy may be applied prior to detection of a malignant tumor.

Normal ras genes are non-tumorigenic and ubiquitous in all mammals. They are highly conserved in evolution and appear to play an important role in maintenance of the cell cycle and normal growth properties. The normal ras protein is a G-protein which binds GTP and has GTPase activity, and is involved in transmitting signals from the external milieu to the inside of the cell, thereby allowing a cell to respond to its environment. Ras\* genes on the other hand alter the normal growth regulation of neoplastic cells by uncoupling cellular behavior from the environment, thus leading to the uncontrolled proliferation of neoplastic cells. Mutation of the ras gene is believed to be an early event in carcinogenesis (Kumar et al., *Science* 248:1101-1104, 1990) which, if treated early, may prevent tumorigenesis.

Ras\* genes occur in a wide variety of cancers, including for example, pancreatic, colon, and lung adenocarcinomas. The spectrum of mutations occurring in the ras\* genes found in a variety of cancers is quite limited. These mutations alter the GTPase activity of the ras protein by converting the normal on/off switch to a constitutive ON position. Tumorigenic mutations in ras\* occur primarily (in vivo) in only 3 codons: 12, 13 and 61. Codon 12 mutations are the most prevalent in both human and animal tumors.

Table 1 below summarizes known in vivo mutations (codons 12, 13 and 61) which activate human ras, as well as potential mutations which have in vitro transforming activity. Potential mutations with in vitro transforming activity were produced by the systematic substitution of amino acids for the normal codon (e.g., other amino acids were substituted for the normal glycine at position 12). In vitro mutations, while not presently known to occur in humans or animals, may serve as the basis for an anti-cancer immunotherapeutic if they are eventually found to arise in vivo.

TABLE 1

#### AMINO ACID SUBSTITUTIONS THAT ACTIVATE HUMAN RAS PROTEINS

Amino Acid	Gly	Gly	Ala	Gln	Glu	Asn	Lys	Asp
Mutant Codon	12	13	59	61	63	116	117	119
In vivo	Val	Asp		Arg				
	Arg	Val		His				
	Asp	Arg		Leu				
	Cys							
	Ala							
	Ser							

TABLE 1-continued

AMINO ACID SUBSTITUTIONS THAT ACTIVATE HUMAN RAS PROTEINS								
In vitro	Phe	Ser	Thr	Val	Lys	His	Glu	His
	Ala			Ala		Ile	Arg	Glu
	Asn			Cys				Ala
	Gln			Asn				Asn
	Glu			Ile				
	His			Met				
	Ile			Thr				
	Leu			Tyr				
	Lys			Trp				
	Met			Phe				
	Phe			Gly				
	Ser							
	Thr							
	Trp							
	Tyr							

Alterations as described above result in the production of proteins containing novel coding sequence(s). The novel proteins encoded by these sequence(s) may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding regions may be utilized to destroy tumorigenic cells containing the altered sequences (ras\*).

Within another embodiment of the present invention, alphavirus vector constructs are provided which direct the expression of an altered p53 (p53\*) gene. Briefly, p53 is a nuclear phosphoprotein which was originally discovered in extracts of transformed cells and thus was initially classified as an oncogene (Linzer and Levine, *Cell* 17:43-52, 1979; Lane and Crawford, *Nature* 278:261-263, 1979). It was later discovered that the original p53 cDNA clones were mutant forms of p53 (Hinds et al., *J. Virol.* 63:739-746, 1989). It now appears that p53 is a tumor suppressor gene which negatively regulates the cell cycle, and that mutation of this gene may lead to tumor formation. Of colon carcinomas that have been studied, 75%-80% show a loss of both p53 alleles, one through deletion and the other through point mutation. Similar mutations are found in lung cancer, and in brain and breast tumors.

The majority of p53 mutations (e.g., p53\*<sup>1</sup>, p53\*<sup>2</sup>, etc.) are clustered between amino acid residues 130 to 290 (see Levine et al., *Nature* 351:453-456, 1991; see also the following references which describe specific mutations in more detail: Baker et al., *Science* 244:217-221, 1989; Nigro et al., *Nature* 342:705-708, 1989 (p53 mutations cluster at four "hot spots" which coincide with the four highly conserved regions of the genes and these mutations are observed in human brain, breast, lung and colon tumors); Vogelstein, *Nature* 348:681-682, 1990; Takahashi et al., *Science* 246:491-494, 1989; Iggo et al., *Lancet* 335:675-679, 1990; James et al., *Proc. Natl. Acad. Sci. USA* 86:2858-2862, 1989; Mackay et al., *Lancet* 11: 1384-1385, 1988; Kelman et al., *Blood* 74:2318-2324, 1989; Malkin et al., *Science* 250:1233-1238, 1990; Baker et al., *Cancer Res.* 50:7717-7722, 1991; Chiba et al., *Oncogene* 5:1603-1610, 1990 (pathogenesis of early stage non-small cell lung cancer is associated with somatic mutations in the p53 gene between codons 132 to 283); Prosser et al., *Oncogene* 5:1573-1579, 1990 (mutations in the p53 gene coding for amino acids 126 through 224 were identified in primary breast cancer); Cheng and Hass, *Mol. Cell. Biol.* 10:5502-5509, 1990; Bartek et al., *Oncogene* 5:893-899, 1990; Rodrigues et al., *Proc. Natl. Acad. Sci. USA* 87:7555-7559, 1990; Menon et al., *Proc. Natl. Acad. Sci. USA* 87:5435-5439, 1990; Mulligan et al., *Proc. Natl. Acad.*

*Sci. USA* 87:5863-5867, 1990; and Romano et al., *Oncogene* 4:1483-1488, 1990 (identification of a p53 mutation at codon 156 in human osteosarcoma derived cell line HOS-SL)).

5 Certain alterations of the p53 gene may be due to certain specific toxins. For example, Bressac et al. (*Nature* 350:429-431, 1991) describes specific G to T mutations in codon 249 in patients affected with hepatocellular carcinoma. One suggested causative agent of this mutation is aflatoxin B<sub>1</sub>, a liver carcinogen which is known to be a food contaminant in Africa.

10 Four regions of the gene that are particularly affected occur at residues 132-145, 171-179, 239-248, and 272-286. Three "hot spots" which are found within these regions that are of particular interest occur at residues 175, 248 and 273 (Levine et al., *Nature* 351:453-456, 1991). These alterations, as well as others which are described above, result in the production of protein(s) which contain novel coding sequence(s). The novel proteins encoded by these sequences may be used as a marker of tumorigenic cells and an immune response directed against these novel coding regions may be utilized to destroy tumorigenic cells containing the altered sequence (p53\*).

20 Once a sequence encoding the altered cellular component has been obtained, it is necessary to ensure that the sequence encodes a non-tumorigenic protein. Various assays which assess the tumorigenicity of a particular cellular component are known and may easily be accomplished. Representative assays include a rat fibroblast assay, tumor formation in nude mice or rats, colony formation in soft agar, and preparation of transgenic animals, such as transgenic mice.

25 Tumor formation in nude mice or rats is a particularly important and sensitive method for determining the tumorigenicity of a particular cellular component. Nude mice lack a functional cellular immune system (i.e., do not possess CTLs), and therefore provide a useful in vivo model in which to test the tumorigenic potential of cells. Normal non-tumorigenic cells do not display uncontrolled growth properties if infected into nude mice. However, transformed cells will rapidly proliferate and generate tumors in nude mice. Briefly, in one embodiment the alphavirus vector construct is administered to syngeneic murine cells, followed by injection into nude mice. The mice are visually examined for a period of 2 to 8 weeks after injection in order to determine tumor growth. The mice may also be sacrificed and autopsied in order to determine whether tumors are present. (Giovannella et al., *J. Natl. Cancer Inst.* 48:1531-1533, 1972; Furesz et al., *Abnormal Cells, New Products and Risk*, Hopps and Petricciani (eds.), Tissue Culture Association, 1985; and Levenbook et al., *J. Biol. Std.* 13:135-141, 1985.)

30 Tumorigenicity may also be assessed by visualizing colony formation in soft agar (Macpherson and Montagnier, *Vir.* 23:291-294, 1964). Briefly, one property of normal non-tumorigenic cells is "contact inhibition" (i.e., cells will stop proliferating when they touch neighboring cells). If cells are plated in a semi-solid agar support medium, normal cells rapidly become contact inhibited and stop proliferating, whereas tumorigenic cells will continue to proliferate and form colonies in soft agar.

35 Transgenic animals, such as transgenic mice, may also be utilized to assess the tumorigenicity of an altered cellular component. (Stewart et al., *Cell* 38:627-637, 1984; Quaife et al., *Cell* 48:1023-1034, 1987; and Koike et al., *Proc. Natl. Acad. Sci. USA* 86:5615-5619, 1989.) In transgenic animals, the gene of interest may be expressed in all tissues of the animal. This dysregulated expression of the transgene may



serve as a model for the tumorigenic potential of the newly introduced gene.

If the altered cellular component is associated with making the cell tumorigenic, then it is necessary to make the altered cellular component non-tumorigenic. For example, within one embodiment the sequence or gene of interest which encodes the altered cellular component is truncated in order to render the gene product non-tumorigenic. The gene encoding the altered cellular component may be truncated to a variety of sizes, although it is preferable to retain as much as possible of the altered cellular component. In addition, it is necessary that any truncation leave intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational termination codons may be introduced downstream of the immunogenic region. Insertion of termination codons will prematurely terminate protein expression, thus preventing expression of the transforming portion of the protein.

Within one embodiment, the ras\* gene is truncated in order to render the ras\* protein non-tumorigenic. Briefly, the carboxy-terminal amino acids of ras\* functionally allow the protein to attach to the cell membrane. Truncation of these sequences renders the altered cellular component non-tumorigenic. Preferably, the ras\* gene is truncated in the purine ring binding site, for example around the sequence which encodes amino acid number 110. The ras\* gene sequence may be truncated such that as little as about 20 amino acids (including the altered amino acid(s)) are encoded by the alphavirus vector construct, although preferably, as many amino acids as possible should be expressed (while maintaining non-tumorigenicity).

Within another embodiment, the p53\* protein is modified by truncation in order to render the cellular component non-tumorigenic. As noted above, not all mutations of the p53 protein are tumorigenic, and therefore, not all mutations would have to be truncated. Nevertheless, within a preferred embodiment, p53\* is truncated to a sequence which encodes amino acids 100 to 300, thereby including all four major "hot spots."

Other altered cellular components which are oncogenic may also be truncated in order to render them non-tumorigenic. For example, both neu and bcr/abl may be truncated in order to render them non-tumorigenic. Non-tumorigenicity may be confirmed by assaying the truncated altered cellular component as described above.

It should be noted, however, that if the altered cellular component is only associated with non-tumorigenic cells in general, and is not required or essential for making the cell tumorigenic, then it is not necessary to render the cellular component non-tumorigenic. Representative examples of such altered cellular components which are not tumorigenic include Rb\*, ubiquitin\*, and mucin\*.

As noted above, in order to generate an appropriate immune response, the altered cellular component must also be immunogenic. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes often possess an immunogenic amphipathic alpha-helix component. In general, however, it is preferable to determine immunogenicity in an assay. Representative assays include an ELISA, which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells such as gamma-interferon assays, IL-2 production assays, and proliferation assays.

As noted above, within another aspect of the present invention, several different altered cellular components may be co-expressed in order to form a general anti-cancer therapeutic. Generally, it will be evident to one of ordinary

skill in the art that a variety of combinations can be made. Within preferred embodiments, this therapeutic may be targeted to a particular type of cancer. For example, nearly all colon cancers possess mutations in ras, p53, DCC APC or MCC genes. An alphavirus vector construct which co-expresses a number of these altered cellular components may be administered to a patient with colon cancer in order to treat all possible mutations. This methodology may also be utilized to treat other cancers. Thus, an alphavirus vector construct which co-expresses mucin\*, ras\*, neu, BRCA1\* and p53\* may be utilized to treat breast cancer.

(b) Antigens from foreign organisms or other pathogens

Within other aspects of the present invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of antigens from foreign organisms or other pathogens. Representative examples of such antigens include bacterial antigens (e.g., *E. coli*, streptococcal, staphylococcal, mycobacterial, etc.), fungal antigens, parasitic antigens, and viral antigens (e.g., influenza virus, Human Immunodeficiency Virus ("HIV"), Hepatitis A, B and C Virus ("HAV", "HBV" and "HCV", respectively), Human Papilloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, TILV I, HTLV II and Cytomegalovirus ("CMV"). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which is capable, under the appropriate conditions, of causing an immune response (i.e., cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen. Cell-mediated immune responses may be mediated through Major Histocompatibility Complex ("MHC") class I presentation, MHC Class II presentation, or both.

Within one aspect of the invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of Hepatitis B antigens. Briefly, the Hepatitis B genome is comprised of circular DNA of about 3.2 kilobases in length and has been well characterized (Tiollais et al., *Science* 213:406-411, 1981; Tiollais et al., *Nature* 317:489-495, 1985; and Ganem and Varmus, *Annu. Rev. Biochem.* 56:651-693, 1987; see also EP 0 278,940, EP 0 241,021, WO 88/10301, and U.S. Pat. Nos. 4,696,898 and 5,024,938, which are hereby incorporated by reference). The Hepatitis B virus presents several different antigens, including among others, three HB "S" antigens (HBsAgs), an HBc antigen (HBcAg), an HBe antigen (HBeAg), and an HBx antigen (HBxAg) (see Blum et al., *TIG* 5(5):154-158, 1989). Briefly, the HBeAg results from proteolytic cleavage of a P22 pre-core intermediate and is secreted from the cell. HBcAg is found in serum as a 17 kD protein. The HBcAg is a protein of 183 amino acids, and the HBxAg is a protein of 145 to 154 amino acids, depending on subtype.

The HBsAgs (designated "large," "middle" and "small") are encoded by three regions of the Hepatitis B genome: S, pre-S2 and pre-S1. The large protein, which has a length varying from 389 to 400 amino acids, is encoded by pre-S1, pre-S2, and S regions, and is found in glycosylated and non-glycosylated forms. The middle protein is 281 amino acids long and is encoded by the pre-S2 and S regions. The small protein is 226 amino acids long and is encoded by the S region. It exists in two forms, glycosylated (GP 27<sup>s</sup>) and non-glycosylated (P24<sup>s</sup>). If each of these regions are expressed separately, the pre-S 1 region will code for a protein of approximately 119 amino acids, the pre-S2 region will code for a protein of approximately 55 amino acids, and the S region will code for a protein of approximately 226 amino acids.



As will be evident to one of ordinary skill in the art, various immunogenic portions of the above-described S antigens may be combined in order to induce an immune response when administered by one of the alphavirus vector constructs described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S open reading frame of HBV, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, epitopes that are found in all human hepatitis B virus S samples are defined as determinant "a". Mutually exclusive subtype determinants, however, have also been identified by two-dimensional double immunodiffusion (Ouchterlony, *Progr. Allergy* 5:1, 1958). These determinants have been designated "d" or "y" and "w" or "r" (LeBouvier, *J. Infect. Dis.* 123:671, 1971; Bancroft et al., *J. Immunol.* 109:842, 1972; and Courouce et al., *Bibl. Haematol.* 42:1-158, 1976). The immunological variability is due to single nucleotide substitutions in two areas of the hepatitis B virus S open reading frame, resulting in the following amino acid changes: (1) exchange of lysine-122 to arginine in the Hepatitis B virus S open reading frame causes a subtype shift from d to y, and (2) exchange of arginine-160 to lysine causes the shift from subtype r to w. In Africans, subtype ayw is predominant, whereas in the U.S. and northern Europe the subtype adw<sub>2</sub> is more abundant (*Molecular Biology of the Hepatitis B Virus*, McLachlan (ed.), CRC Press, 1991). As will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for administration which is appropriate to the particular hepatitis B virus subtype which is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, preferably, by sequencing the S open reading frame of HBV virus isolated from individuals within that region.

Also presented by HBV are pol ("HBV pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected livers. The polymerase protein consists of at least two domains: the amino terminal domain which encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of HBV pol may be determined utilizing methods described herein (e.g., below and in Example 13), utilizing alphavirus vector constructs described below, and administered in order to generate an immune response within a warm-blooded animal. Similarly, other HBV antigens, such as ORF 5 and ORF 6 (Miller et al., *Hepatology* 9:322-327, 1989) may be expressed utilizing alphavirus vector constructs as described herein. Representative examples of alphavirus vector constructs utilizing ORF 5 and ORF 6 are set forth below in the examples.

As noted above, at least one immunogenic portion of a hepatitis B antigen is incorporated into an alphavirus vector construct. The immunogenic portion(s) which are incorporated into the alphavirus vector construct may be of varying length, although it is generally preferred that the portions be at least 9 amino acids long and may include the entire antigen. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes may be predicted utilizing computer algorithms such as TSITES (MedImmune, Maryland), in order to scan coding regions for potential T-helper sites and CTL sites. From this analysis, peptides are synthesized and used as targets in an in vitro cytotoxic assay. Other assays, however, may also be utilized, including, for example, ELISA, which detects the presence

of antibodies against the newly introduced vector, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays and proliferation assays.

Immunogenic portions may also be selected by other methods. For example, the HLA A2.1 transgenic mouse has been shown to be useful as a model for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T cell receptor repertoire recognizes the same antigenic determinants recognized by human T cells. In both systems, the CTL response generated in the HLA A2.1 transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al., *J. Exp. Med.* 173:1007-1015, 1991; Vitiello et al., *Abstract of Molecular Biology of Hepatitis B Virus Symposia*, 1992).

Particularly preferred immunogenic portions for incorporation into alphavirus vector constructs include HBeAg, HBcAg and HBsAg, as described in greater detail below in Example 13.

Additional immunogenic portions of the hepatitis B virus may be obtained by truncating the coding sequence at various locations including, for example, the following sites: Bst UI, Ssp I, Ppu M1, and Msp I (Valenzuela et al., *Nature* 280:815-19, 1979; Valenzuela et al., *Animal Virus Genetics: ICN/UCLA Symp. Mol. Cell Biol.*, 1980, B. N. Fields and R. Jaenisch (eds.), pp. 57-70, New York: Academic). Further methods for determining suitable immunogenic portions as well as methods are also described below in the context of hepatitis C.

As noted above, more than one immunogenic portion may be incorporated into the alphavirus vector construct. For example, an alphavirus vector construct may express (either separately or as one construct) all or immunogenic portions of HBcAg, HBeAg, HBsAg, HBxAg, as well as immunogenic portions of HCV antigens.

#### 7. SOURCES FOR HETEROLOGOUS SEQUENCES

Sequences which encode the above-described proteins may be readily obtained from a variety of sources, including for example, depositories such as the American Type Culture Collection (ATCC, Rockville, Md.), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford, England). Representative examples include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids); BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contain sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No. 67024 (which contain a sequence which encodes Interleukin-1b); ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2); ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3); ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6).

Sequences which encode altered cellular components as described above may be readily obtained from a variety of sources. For example, plasmids which contain sequences that encode altered cellular products may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Md.), or from commercial sources such as Advanced Biotechnologies (Columbia, Md.). Representative examples of plasmids containing some of the above-described sequences include ATCC No. 41000 (containing a

G to T mutation in the 12th codon of ras), and ATCC No. 41049 (containing a G to A mutation in the 12th codon).

Alternatively, plasmids which encode normal cellular components may also be obtained from depositories such as the ATCC (see, for example, ATCC No. 41001, which contains a sequence which encodes the normal ras protein; ATCC No. 57103, which encodes abl; and ATCC Nos. 59120 or 59121, which encode the bcr locus) and mutated to form the altered cellular component. Methods for mutagenizing particular sites may readily be accomplished using methods known in the art (see Sambrook et al., supra., 15.3 et seq.). In particular, point mutations of normal cellular components such as ras may readily be accomplished by site-directed mutagenesis of the particular codon, for example, codons 12, 13 or 61.

Sequences which encode the above-described viral antigens may likewise be obtained from a variety of sources. For example, molecularly cloned genomes which encode the hepatitis B virus may be obtained from sources such as the American Type Culture Collection (ATCC, Rockville, Md.). For example, ATCC No. 45020 contains the total genomic DNA of hepatitis B (extracted from purified Dane particles) (see FIG. 3 of Blum et al., *TIG* 5(5):154-158, 1989) in the Bam HI site of pBR322 (Moriarty et al., *Proc. Natl. Acad. Sci. USA* 78:2606-2610, 1981).

Alternatively, cDNA sequences which encode the above-described heterologous sequences may be obtained from cells which express or contain the sequences. Briefly, within one embodiment, mRNA from a cell which expresses the gene of interest is reverse transcribed with reverse transcriptase using oligonucleotide dT or random primers. The single stranded cDNA may then be amplified by PCR (see U.S. Pat. Nos. 4,683,202; 4,683,195 and 4,800,159. See also *PCR Technology: Principles and Applications for DNA Amplification*, Erlich (ed.), Stockton Press, 1989) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double-stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence-specific DNA primers, dATP, dCTP, dGTP and dTTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Sequences which encode the above-described proteins may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (e.g., APB DNA synthesizer model 392 (Foster City, Calif.)).

#### F. EUKARYOTIC LAYERED VECTOR INITIATION SYSTEMS

Due to the size of a full-length genomic alphavirus cDNA clone, in vitro transcription of full-length RNA molecules is rather inefficient. This results in a lowered transfection efficiency, in terms of infectious centers of virus (as measured by plaque formation), relative to the amount of in vitro transcribed RNA transfected. Such inefficiency is also relevant to the in vitro transcription of alphavirus expression vectors. Testing of candidate cDNA clones and other alphavirus cDNA expression vectors for their ability to initiate an infectious cycle or to direct the expression of a heterologous sequence would thus be greatly facilitated if a cDNA clone was transfected into susceptible cells as a DNA molecule, which then directed the synthesis of viral RNA in vivo.

Therefore, within one aspect of the present invention DNA-based vectors (referred to as "Eukaryotic Layered Vector Initiation Systems") are provided which are capable of directing the synthesis of viral RNA in vivo. In particular,

eukaryotic layered vector initiation systems are provided comprising a promoter which is capable of initiating the 5' synthesis of RNA from cDNA, a construct which is capable of autonomous replication in a cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription termination. Briefly, such eukaryotic layered vector initiation systems provide a two-stage or "layered" mechanism which controls expression of heterologous nucleotide sequences. The first layer initiates transcription of the second layer, and comprises a promoter which is capable of initiating the 5' synthesis of RNA from cDNA (e.g., a 5' promoter), a 3' transcription termination site, as well as one or more splice sites and/or a polyadenylation site, if desired. Representative promoters suitable for use within the present invention include both eukaryotic (e.g., pol I, II, or III) and prokaryotic promoters, and inducible or non-inducible (i.e., constitutive) promoters, such as, for example, Murine Leukemia virus promoters (e.g., MoMLV), metallothionein promoters, the glucocorticoid promoter, Drosophila actin 5C distal promoter, SV 40 promoter, heat shock protein 65 promoter, heat shock protein 70 promoter, immunoglobulin promoters, Mouse polyoma virus promoter ("Py"), rous sarcoma virus ("RSV"), BK virus and JC virus promoters, MMTV promoter, alphavirus junction region, CMV promoter, Adenovirus VA1RNA, rRNA promoter, tRNA methionine promoter, CaMV 35S promoter, nopaline synthetase promoter, and the lac promoter. The second layer comprises a vector construct which is capable of expressing one or more heterologous nucleotide sequences and of replication in a cell, either autonomously or in response to one or more factors. Within one embodiment of the invention, the second layer construct may be an alphavirus vector construct as described above.

A wide variety of vector systems may be utilized as the first layer of the eukaryotic layered vector initiation system, including for example, viral vector constructs developed from DNA viruses such as those classified in the Poxviridae, including for example canary pox virus or vaccinia virus (e.g., Fisher-Hoch et al., *PNAS* 86:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci.* 569:86-103, 1989; Flexner et al., *Vaccine* 8:17-21, 1990; U.S. Pat. Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); Papoviridae such as BKV, JCV or SV40 (e.g., Mulligan et al., *Nature* 277:108-114, 1979); Adenoviridae, such as adenovirus (e.g., Berkner, *Biotechniques* 6:616-627, 1988; Rosenfeld et al., *Science* 252:431-434, 1991); Parvoviridae, such as adeno-associated virus (e.g., Samulski et al., *J. Vir.* 63:3822-3828, 1989; Mendelson et al., *Virol.* 166:154-165, 1988; PA 7/222,684); Herpesviridae, such as Herpes Simplex Virus (e.g., Kit, *Adv. Exp. Med. Biol.* 215:219-236, 1989); and Hepadnaviridae (e.g., HBV), as well as certain RNA viruses which replicate through a DNA intermediate, such as the Retroviridae (see, e.g., U.S. Pat. No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805; Retroviridae include leukemia in viruses such as MoMLV and immunodeficiency viruses such as HIV, e.g., Poznansky, *J. Virol.* 65:532-536, 1991).

Similarly, a wide variety of vector systems may be utilized as second layer of the eukaryotic layered vector initiation system, including for example, vector systems derived from viruses of the following families: Picornaviridae (e.g., poliovirus, rhinovirus, coxsackieviruses), Caliciviridae, Togaviridae (e.g. alphavirus, rubella), Flaviviridae (e.g., yellow fever), Coronaviridae (e.g., HCV, TGEV, IBV, MHV, BCoV), Bunyaviridae, Arenaviridae, Retroviridae (e.g., RSV, MoMLV, HIV, HTLV), hepatitis delta virus and Astrovirus. In addition, non-mammalian RNA

viruses (as well as components derived therefrom) may also be utilized, including for example, bacterial and bacteriophage replicases, as well as components derived from plant viruses, such as potexviruses (e.g., PVX), carlaviruses (e.g., PVM), tobnaviruses (e.g., TRV, PEBV, PRV), Tobamoviruses (e.g., TMV, ToMV, PPMV), luteoviruses (e.g., PLRV), potyviruses (e.g., TEV, PPV, PVY), tombusviruses (e.g., CyRSV), nepoviruses (e.g., GFLV), bromoviruses (e.g., BMV), and topamoviruses.

The replication competency of the autocatalytic vector construct, contained within the second layer of the eukaryotic vector initiation system, may be measured by a variety of assays known to one of skill in the art including, for example, ribonuclease protection assays which measure increases in both positive-sense and negative-sense RNA over time, in transfected cells, in the presence of an inhibitor of cellular RNA synthesis, such as dactinomycin, and assays which measure the synthesis of a subgenomic RNA or expression of a heterologous reporter gene in transfected cells.

Within particularly preferred embodiments of the invention, eukaryotic layered vector initiation systems are provided that comprise a 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA, followed by a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus nonstructural proteins, a viral junction region which is either active or which has been inactivated such that viral transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription termination. Within various embodiments, the viral junction region may be modified, such that viral transcription of the subgenomic fragment is merely reduced, rather than inactivated. Within other embodiments, a second viral junction region may be inserted following the first inactivated viral junction region, the second viral junction region being either active or modified such that viral transcription of the subgenomic fragment is reduced.

Following transcription of an alphavirus cDNA vector construct, the resulting alphavirus RNA vector molecule is comprised of a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus nonstructural proteins, a viral junction region, a heterologous nucleotide sequence, an alphavirus RNA polymerase recognition sequence, and a polyadenylate sequence.

Various aspects of the alphavirus cDNA vector constructs have been discussed above, including the 5' sequence which is capable of initiating transcription of an alphavirus, the nucleotide sequence encoding alphavirus nonstructural proteins, the viral junction region, including junction regions which have been inactivated such that viral transcription of the subgenomic fragment is prevented, and the alphavirus RNA polymerase recognition sequence. In addition, modified junction regions and tandem junction regions have also been discussed above.

Within certain aspects of the present invention, methods are provided for delivering a heterologous nucleotide sequence to a warm-blooded animal, comprising the step of administering a eukaryotic layered vector initiation system as described above, to a warm-blooded animal. Eukaryotic layered vector initiation systems may be administered to warm-blooded animals either directly (e.g., intravenously, intramuscularly, intraperitoneally, subcutaneously, orally, rectally, intraocularly, intranasally), or by various physical methods such as lipofection (Felgner et al., *Proc. Natl. Acad.*

*Sci. USA* 84:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature* 352:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991); liposomes of several types (see, e.g., Wang et al., *PNAS* 84:7851-7855, 1987);  $\text{CaPO}_4$  (Dubensky et al., *PNAS* 81:7529-7533, 1984); DNA ligand (Wu et al., *J. of Biol. Chem.* 264:16985-16987, 1989); administration of nucleic acids alone (WO 90/11092); or administration of DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther.* 3:147-154, 1992); via polycation compounds such as polylysine, utilizing receptor specific ligands; as well as with psoralen inactivated viruses such as Sendai or Adenovirus. In addition, the eukaryotic layered vector initiation systems may either be administered directly (i.e., in vivo), or to cells which have been removed (ex vivo), and subsequently returned.

Eukaryotic layered vector initiation systems may be administered to a warm-blooded animal for any of the therapeutic uses described herein, including for example, for the purpose of stimulating a specific immune response; inhibiting the interaction of an agent with a host cell receptor; to express a toxic palliative, including for example, conditional toxic palliatives; to immunologically regulate the immune system; to express markers, and for replacement gene therapy. These and other uses are discussed in more detail below.

In another embodiment of this aspect of the invention, eukaryotic layered vector initiation systems can be utilized to direct the expression of one or more recombinant proteins by eukaryotic cells. As used herein, a "recombinant protein" refers to a protein, polypeptide, enzyme, or fragment thereof. Using this approach, proteins having therapeutic or other commercial application can be more cost-effectively produced. Furthermore, proteins produced in eukaryotic cells may be post-translationally modified (e.g., glycosylated, sulfated, acetylated, etc.), as compared to proteins produced in prokaryotic cells. In addition, such systems may be employed in the in vivo production of various chemical compounds, e.g., fine or specialty chemicals.

Within this embodiment, a eukaryotic layered vector initiation system encoding the desired protein, enzyme, or enzymatic pathway (as may be required for the production of a desired chemical) is transformed, transfected, or otherwise introduced into a suitable eukaryotic cell line. Representative examples of proteins which can be produced using such a system include, but are not limited to, insulin (see U.S. Pat. No. 4,431,740 and BE 885196A), hemoglobin (Lawn et al. *Cell* 21:647-51, 1980), erythropoietin (EPO; see U.S. Pat. No. 4,703,008), megakaryocyte growth and differentiation factor (MGDF), stem cell factor (SCF), G-CSF (Nagata et al. *Nature* 319:415-418, 1986), GM-CSF, M-CSF (see WO 8706954), the *flt3* ligand (Lyman, et al. (1993), *Cell*, vol. 75, pp. 1157-1167), EGF, acidic and basic FGF, PDGF, members of the interleukin or interferon families, supra, neurotropic factors (e.g., BDNF; Rosenthal et al. *Endocrinology* 129:1289-1294, 1991, NT-3; see WO 9103569, CNTF; see WO 9104316, NGF; see WO 9310150), coagulation factors (e.g., factors VIII and IX), thrombolytic factors such as t-PA (see EP 292009, AU 8653302 and EP 174835) and streptokinase (see EP 407942), human growth hormone (see JP 94030582 and U.S. Pat. No. 4,745,069) and other animal somatotropins, and integrins and other cell adhesion molecules, such as ICAM-1 and ELAM. Genes encoding such recombinant proteins are among the heterologous nucleic acid sequences of the invention. As those in the art will appreciate, once

characterized, any gene can be readily cloned into a eukaryotic layered vector initiation system according to the invention, followed by introduction into a suitable host cell and expression of the desired gene.

In a preferred embodiment of this and other aspects of the invention, the eukaryotic layered vector initiation system is one derived from an alphavirus vector, such as a Sindbis vector construct, which has been adapted to replicate in one or more cell lines from a particular eukaryotic species, especially a mammalian species, such as humans. For instance, if the gene encoding the recombinant protein to be expressed is of human origin and the protein is intended for human therapeutic use, production in a suitable human cell line may be preferred in order that the protein be post-translationally modified as would be expected to occur in humans. This approach may be useful in further enhancing recombinant protein production. Given the overall plasticity of an alphaviral genome due to the infidelity of the viral replicase, variant strains with an enhanced ability to establish high titer productive infection in selected eukaryotic cells (e.g., human, murine, canine, feline, etc.) can be isolated. Additionally, variant alphaviral strains having an enhanced ability to establish high titer persistent infection in eukaryotic cells may also be isolated using this approach. Alphavirus expression vectors can then be constructed from cDNA clones of these variant strains according to procedures provided herein.

Within another preferred embodiment of this aspect of the invention, the eukaryotic layered vector initiation system comprises a promoter for initial alphaviral vector transcription that is transcriptionally active only in a differentiated cell type. It is well established that alphaviral infection of cells in culture, in particular those derived from hamster (e.g., baby hamster kidney cells) or chicken (e.g., chicken embryo fibroblasts), may result in cytotoxicity. Thus, to produce a stably transformed or transfected host cell line, the eukaryotic layered vector initiation system is preferably introduced into a host cell wherein the promoter which enables the initial vector amplification is a transcriptionally inactive, but inducible, promoter. In a particularly preferred embodiment, such a promoter is differentiation state dependent. In this configuration, activation of the promoter and subsequent activation of the alphavirus DNA vector coincides with induction of cell differentiation. Upon growth to a certain cell number of such a stably transformed or transfected host cell line, the appropriate differentiation stimulus is provided, thereby initiating transcription of the vector construct and amplified expression of the desired gene and encoded polypeptide(s). Many such differentiation state-dependent promoters are known to those in the art, as are cell lines which can be induced to differentiate by application of a specific stimulus. Representative examples include cell lines F9 and P19, HL60, and Freund erythroleukemic cell lines and HEL, which are activated by retinoic acid, horse serum, and DMSO, respectively.

#### G. ALPHAVIRUS PACKAGING CELL LINES

Within further embodiments of the invention, alphavirus packaging cell lines are provided. In particular, within one aspect of the present invention, alphavirus packaging cell lines are provided wherein the viral structural proteins, supplied in trans from one or more stably integrated expression vectors, are able to encapsidate transfected, transduced, or intracellularly produced vector RNA transcripts in the cytoplasm and release infectious packaged vector particles through the cell membrane, thus creating an alphavirus vector producing cell line. Alphavirus RNA vector molecules, capable of replicating in the cytoplasm of the

packaging cell, can be produced initially utilizing, for example, an SP6 RNA polymerase system to transcribe in vitro a cDNA vector clone encoding the gene of interest and the alphavirus nonstructural proteins (described previously). Vector RNA transcripts are then transfected into the alphavirus packaging cell line, such that the vector RNA replicates to high levels, and is subsequently packaged by the viral structural proteins, yielding infectious vector particles. Because of the extended length of the alphavirus cDNA molecule, the in vitro transcription process is inefficient. Further, only a fraction of the cells contained in a monolayer are typically transfected by most procedures.

In an effort to optimize vector producing cell line performance and titer, two successive cycles of gene transfer may be performed. In particular, rather than directly transfecting alphavirus RNA vector molecules into the final producing cell line, the vector may first be transfected into a primary alphavirus packaging cell line. The transfected primary packaging cell line releases infectious vector particles into the culture supernatants and these vector-containing supernatants are subsequently used to transduce a fresh monolayer of alphavirus packaging cells. Transduction into the final alphavirus vector producing cells is preferred over transfection because of its higher RNA transfer efficiency into cells and optimized biological placement of the vector in the cell. This leads to higher expression and higher titer of packaged infectious recombinant alphavirus vector.

Within certain embodiments of the invention, alphavirus vector particles may fail to transduce the same packaging cell line because the cell line produces extracellular envelope proteins which block cellular receptors for alphavirus vector particle attachment, a second type of alphavirus vector particle is generated which maintains the ability to transduce the alphavirus packaging cells. This second type of viral particle is produced by a packaging cell line known as a "hopping cell line," which produces transient vector particles as the result of being transfected with in vitro transcribed alphavirus RNA vector transcripts. Briefly, the hopping cell line is engineered to redirect the receptor tropism of the transiently produced vector particles by providing alternative viral envelope proteins which redirect alphavirus vectors to different cellular receptors, in a process termed pseudotyping. Two primary approaches have been devised for alphavirus vector particle pseudotyping. The first approach consists of an alphavirus packaging cell line expressing the vesicular stomatitis virus G protein (VSV-G). The second approach for producing a pseudotyped alphavirus vector particle is to use currently available retroviral packaging cell lines containing retroviral gag/pol and env sequences which would be capable of packaging an alphavirus RNA vector containing a retroviral packaging sequence (e.g., WO 92/05266).

Within other embodiments of the invention, a second approach has also been devised in which a stably integrated DNA expression vector is used to produce the alphavirus vector RNA molecule, which, as in the first approach, maintains the autocatalytic ability to self-replicate. This approach allows for continued vector expression over extended periods of culturing because the integrated DNA vector expression system is maintained through a drug selection marker and the DNA system will constitutively express unaltered RNA vectors which cannot be diluted out by defective RNA copies. In this "alphavirus producer cell line" configuration, the DNA-based alphavirus vector is introduced initially into the packaging cell line by transfection, since size restrictions could prevent packaging of the expression vector into a viral vector particle for

transduction. Also, for this configuration, the SP6 RNA polymerase recognition site of the plasmid, previously used to transcribe vector RNA in vitro, is replaced with another appropriate promoter sequence defined by the parent cell line used. In addition, this plasmid sequence also contains a selection marker different from that used to create the packaging cell line.

The expression of alphavirus proteins and/or vector RNA above certain levels may result in cytotoxic effects in packaging cell lines. Therefore, within certain embodiments of the invention, it may be desirable for these elements to be expressed only after the packaging/producer cells have been propagated to a certain critical density. For this purpose, additional packaging or producer cell line modifications are made whereby the structural proteins necessary for packaging are synthesized only after induction by the RNA vector itself or some other stimulus. Also, other modifications allow for the individual expression of these proteins under the control of separate inducible elements, by utilizing expression vectors which unlink the genes encoding these proteins. In addition, expression of the integrated vector molecule itself, in some instances, is controlled by yet another inducible system. This configuration results in a cascade of events following induction, that ultimately leads to the production of packaged vector particles.

#### H. METHODS FOR UTILIZING ALPHAVIRUS VECTORS

##### 1. IMMUNOSTIMULATION

Within other aspects of the present invention, compositions and methods are provided for administering an alphavirus vector construct which is capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous, auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV HTLV I, HTLV II, CMV, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease.

More specifically, within one aspect of the present invention, compositions and methods are provided for stimulating an immune response (either humoral or cell-mediated) to a pathogenic agent, such that the pathogenic agent is either killed or inhibited. Representative examples of pathogenic agents include bacteria, fungi, parasites, viruses and cancer cells.

Within one embodiment of the invention the pathogenic agent is a virus, and methods are provided for stimulating a specific immune response and inhibiting viral spread by using recombinant alphavirus viral particles designed to deliver a vector construct that directs the expression of an antigen or modified form thereof to susceptible target cells capable of either (1) initiating an immune response to the viral antigen or (2) preventing the viral spread by occupying cellular receptors required for viral interactions. Expression of the vector nucleic acid encoded protein may be transient or stable with time. Where an immune response is to be stimulated to a pathogenic antigen, the recombinant alphavirus is preferably designed to express a modified form of the antigen which will stimulate an immune response and which has reduced pathogenicity relative to the native antigen. This immune response is achieved when cells present antigens in the correct manner, i.e., in the context of the MHC class I and/or II molecules along with accessory molecules such as CD3, ICAM-1, ICAM-2, LFA-1, or analogues thereof (e.g., Altmann et al., *Nature* 338:512, 1989). Cells infected with alphavirus vectors are expected to do this efficiently because they closely mimic genuine viral infection and because they: (a) are able to infect non-replicating cells, (b) do not

integrate into the host cell genome, (c) are not associated with any life threatening diseases, and (d) express high levels of heterologous protein. Because of these differences, alphavirus vectors can easily be thought of as safe viral vectors which can be used on healthy individuals for vaccine use.

This aspect of the invention has a further advantage over other systems that might be expected to function in a similar manner, in that the presenter cells are fully viable and healthy, and low levels of viral antigens, relative to heterologous genes, are expressed. This presents a distinct advantage since the antigenic epitopes expressed can be altered by selective cloning of sub-fragments of the gene for the antigen into the recombinant alphavirus, leading to responses against immunogenic epitopes which may otherwise be overshadowed by immunodominant epitopes. Such an approach may be extended to the expression of a peptide having multiple epitopes, one or more of the epitopes being derived from different proteins. Further, this aspect of the invention allows efficient stimulation of cytotoxic T lymphocytes (CTL) directed against antigenic epitopes, and peptide fragments of antigens encoded by sub-fragments of genes, through intracellular synthesis and association of these peptide fragments with MHC Class I molecules. This approach may be utilized to map major immunodominant epitopes for CTL induction.

An immune response may also be achieved by transferring to an appropriate immune cell (such as a T lymphocyte) the gene for the specific T cell receptor which recognizes the antigen of interest (in the context of an appropriate MHC molecule if necessary), for an immunoglobulin which recognizes the antigen of interest, or for a hybrid of the two which provides a CTL response in the absence of the MHC context. Thus, the recombinant alphavirus infected cells may be used as an immunostimulant, immunomodulator, or vaccine.

In another embodiment of the invention, methods are provided for producing inhibitor palliatives wherein alphavirus vectors deliver and express defective interfering viral structural proteins, which inhibit viral assembly. Such vectors may encode defective gag, pol, env or other viral particle proteins or peptides and these would inhibit in a dominant fashion the assembly of viral particles. This occurs because the interaction of normal subunits of the viral particle is disturbed by interaction with the defective subunits.

In another embodiment of the invention, methods are provided for the expression of inhibiting peptides or proteins specific for viral protease. Briefly, viral protease cleaves the viral gag and gag/pol proteins into a number of smaller peptides. Failure of this cleavage in all cases leads to complete inhibition of production of infectious retroviral particles. As an example, the HIV protease is known to be an aspartyl protease and these are known to be inhibited by peptides made from amino acids from protein or analogues. Vectors to inhibit HIV will express one or multiple fused copies of such peptide inhibitors.

Another embodiment involves the delivery of suppressor genes which, when deleted, mutated, or not expressed in a cell type, lead to tumorigenesis in that cell type. Reintroduction of the deleted gene by means of a viral vector leads to regression of the tumor phenotype in these cells. Examples of such cancers are retinoblastoma and Wilms Tumor. Since malignancy can be considered to be an inhibition of cellular terminal differentiation compared with cell growth, the alphavirus vector delivery and expression of gene products which lead to differentiation of a tumor should also, in general, lead to regression.

In yet another embodiment, the alphavirus vector provides a therapeutic effect by transcribing a ribozyme (an RNA enzyme) (Haseloff and Gerlach, *Nature* 334:585, 1989) which will cleave and hence inactivate RNA molecules corresponding to a pathogenic function. Since ribozymes function by recognizing a specific sequence in the target RNA and this sequence is normally 12 to 17 bp, this allows specific recognition of a particular RNA species such as a RNA or a retroviral genome. Additional specificity may be achieved in some cases by making this a conditional toxic

palliative (see below). One way of increasing the effectiveness of inhibitory palliatives is to express viral inhibitory genes in conjunction with the expression of genes which increase the probability of infection of the resistant cell by the virus in question. The result is a nonproductive "dead-end" event which would compete for productive infection events. In the specific case of HIV, vectors may be delivered which inhibit HIV replication (by expressing anti-sense tat, etc., as described above) and also overexpress proteins required for infection, such as CD4. In this way, a relatively small number of vector-infected HIV-resistant cells act as a "sink" or "magnet" for multiple nonproductive fusion events with free virus or virally infected cells.

## 2. BLOCKING AGENTS

Many infectious diseases, cancers, autoimmune diseases, and other diseases involve the interaction of viral particles with cells, cells with cells, or cells with factors. In viral infections, viruses commonly enter cells via receptors on the surface of susceptible cells. In cancers, cells may respond inappropriately or not at all to signals from other cells or factors. In autoimmune disease, there is inappropriate recognition of "self" markers. Within the present invention, such interactions may be blocked by producing, in vivo, an analogue to either of the partners in an interaction.

This blocking action may occur intracellularly, on the cell membrane, or extracellularly. The blocking action of a viral or, in particular, an alphavirus vector carrying a gene for a blocking agent, can be mediated either from inside a susceptible cell or by secreting a version of the blocking protein to locally block the pathogenic interaction.

In the case of HIV, the two agents of interaction are the gp 120/gp 41 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker would be a vector construct expressing either an HIV env analogue that blocks HIV entry without causing pathogenic effects, or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp 41 is secreted or produced only intracellularly so as to protect only the vector-containing cell. It may be advantageous to add human immunoglobulin heavy chains or other components to CD4 in order to enhance stability or complement lysis. Delivery of an alphavirus vector encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule. Efficacy of treatment can be assayed by measuring the usual indicators of disease progression, including antibody level, viral antigen production, infectious HIV levels, or levels of nonspecific infections.

## 3. EXPRESSION OF PALLIATIVES

Techniques similar to those described above can be used to produce recombinant alphavirus vector constructs which direct the expression of an agent (or "palliative") which is capable of inhibiting a function of a pathogenic agent or gene. Within the present invention, "capable of inhibiting a function" means that the palliative either directly inhibits the function or indirectly does so, for example, by converting an

agent present in the cells from one which would not normally inhibit a function of the pathogenic agent to one which does. Examples of such functions for viral diseases include adsorption, replication, gene expression, assembly, and exit of the virus from infected cells. Examples of such functions for a cancerous cell or cancer-promoting growth factor include viability, cell replication, altered susceptibility to external signals (e.g., contact inhibition), and lack of production or production of mutated forms of anti-oncogene proteins.

### (a) Inhibitor Palliatives

In one aspect of the present invention, the alphavirus vector construct directs the expression of a gene which can interfere with a function of a pathogenic agent, for instance in viral or malignant diseases. Such expression may either be essentially continuous or in response to the presence in the cell of another agent associated either with the pathogenic condition or with a specific cell type (an "identifying agent"). In addition, vector delivery may be controlled by targeting vector entry specifically to the desired cell type (for instance, a virally infected or malignant cell) as discussed above.

One method of administration is leukaphoresis, in which about 20% of an individual's PBLs are removed at any one time and manipulated in vitro. Thus, approximately  $2 \times 10^9$  cells may be treated and replaced. Repeat treatments may also be performed. Alternatively, bone marrow may be treated and allowed to amplify the effect as described above. In addition, packaging cell lines producing a vector may be directly injected into a subject, allowing continuous production of recombinant virions.

In one embodiment, alphavirus vectors which express RNA complementary to key pathogenic gene transcripts (for example, a viral gene product or an activated cellular oncogene) can be used to inhibit translation of that transcript into protein, such as the inhibition of translation of the HIV tat protein. Since expression of this protein is essential for viral replication, cells containing the vector would be resistant to HIV replication.

In a second embodiment, where the pathogenic agent is a single-stranded virus having a packaging signal, RNA complementary to the viral packaging signal (e.g., an HIV packaging signal when the palliative is directed against HIV) is expressed, so that the association of these molecules with the viral packaging signal will, in the case of retroviruses, inhibit stem loop formation or tRNA primer binding required for proper encapsidation or replication of the alphavirus RNA genome.

In a third embodiment, an alphavirus vector may be introduced which expresses a palliative capable of selectively inhibiting the expression of a pathogenic gene, or a palliative capable of inhibiting the activity of a protein produced by the pathogenic agent. In the case of HIV, one example is a mutant tat protein which lacks the ability to transactivate expression from the HIV LTR and interferes (in a transdominant manner) with the normal functioning of tat protein. Such a mutant has been identified for HTLV II tat protein ("XII Leu<sup>3</sup>" mutant; see Wachsmann et al., *Science* 235:674, 1987). A mutant transrepressor tat should inhibit replication much as has been shown for an analogous mutant repressor in HSV-1 (Friedmann et al., *Nature* 335:452, 1988).

Such a transcriptional repressor protein may be selected for in tissue culture using any viral-specific transcriptional promoter whose expression is stimulated by a virus-specific transactivating protein (as described above). In the specific case of HIV, a cell line expressing HIV tat protein and the

HSVTK gene driven by the HIV promoter will die in the presence of ACV. However, if a series of mutated tat genes are introduced to the system, a mutant with the appropriate properties (i.e., represses transcription from the HIV promoter in the presence of wild-type tat) will grow and be selected. The mutant gene can then be reisolated from these cells. A cell line containing multiple copies of the conditionally lethal vector/tat system may be used to assure that surviving cell clones are not caused by endogenous mutations in these genes. A battery of randomly mutagenized tat genes are then introduced into these cells using a "rescuable" alphavirus vector (i.e., one that expresses the mutant tat protein and contains a bacterial origin of replication and drug resistance marker for growth and selection in bacteria). This allows a large number of random mutations to be evaluated and permits facile subsequent molecular cloning of the desired mutant cell line. This procedure may be used to identify and utilize mutations in a variety of viral transcriptional activator/viral promoter systems for potential antiviral therapies.

#### 4. CONDITIONAL TOXIC PALLIATIVES

Another approach for inhibiting a pathogenic agent is to express a palliative which is toxic for the cell expressing the pathogenic condition. In this case, expression of the palliative from the vector should be limited by the presence of an entity associated with the pathogenic agent, such as a specific viral RNA sequence identifying the pathogenic state, in order to avoid destruction of nonpathogenic cells.

In one embodiment of this method, a recombinant alphavirus vector carries a vector construct containing a toxic gene (as discussed above) expressed from a cell-specific responsive vector. In this manner, rapidly replicating cells, which contain the RNA sequences capable of activating the cell-specific responsive vectors, are preferentially destroyed by the cytotoxic agent produced by the alphavirus vector construct.

In a similar manner to the preceding embodiment, the alphavirus vector construct can carry a gene for phosphorylation, phosphoribosylation, ribosylation, or other metabolism of a purine- or pyrimidine-based drug. This gene may have no equivalent in mammalian cells and might come from organisms such as a virus, bacterium, fungus, or protozoan. An example of this would be the *E. coli* guanine phosphoribosyl transferase gene product, which is lethal in the presence of thioxanthine (see Besnard et al., *Mol. Cell Biol.* 7:4139-4141, 1987). Conditionally lethal gene products of this type (also referred to as "pro-drugs" above) have application to many presently known purine- or pyrimidine-based anticancer drugs, which often require intracellular ribosylation or phosphorylation in order to become effective cytotoxic agents. The conditionally lethal gene product could also metabolize a nontoxic drug which is not a purine or pyrimidine analogue to a cytotoxic form (see Searle et al., *Brit. J. Cancer* 53:377-384, 1986).

Mammalian viruses in general tend to have "immediate early" genes which are necessary for subsequent transcriptional activation from other viral promoter elements. RNA sequences of this nature are excellent candidates for activating alphavirus vectors intracellular signals (or "identifying agents") of viral infection. Thus, conditionally lethal genes expressed from alphavirus cell-specific vectors responsive to these viral "immediate early" gene products could specifically kill cells infected with any particular virus. Additionally, since the human and interferon promoter elements are transcriptionally activated in response to infection by a wide variety of nonrelated viruses, the introduction of vectors expressing a conditionally lethal gene product like

HSVTK, for example, in response to interferon production could result in the destruction of cells infected with a variety of different viruses.

In another aspect of the present invention, the recombinant alphavirus viral vector carries a vector construct that directs the expression of a gene product capable of activating an otherwise inactive precursor into an active inhibitor of the pathogenic agent. For example, the HSVTK gene product may be used to more effectively metabolize potentially antiviral nucleoside analogues such as AZT or ddC. The HSVTK gene may be expressed under the control of a cell-specific responsive vector and introduced into these cell types. AZT (and other nucleoside antivirals) must be metabolized by cellular mechanisms to the nucleotide triphosphate form in order to specifically inhibit retroviral reverse transcriptase, and thus, HIV replication (Furman et al., *Proc. Natl. Acad. Sci. USA* 83:8333-8337, 1986). Constitutive expression of HSVTK (a nucleoside and nucleoside kinase with very broad substrate specificity) results in more effective metabolism of these drugs to their biologically active nucleotide triphosphate form. AZT or ddC therapy will thereby be more effective, allowing lower doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, will be made more efficacious.

Administration of these alphavirus vectors to human T cell and macrophage/monocyte cell lines can increase their resistance to HIV in the presence of AZT and ddC compared to the same cells without retroviral vector treatment. Treatment with AZT would be at lower than normal levels to avoid toxic side effects but still efficiently inhibit the spread of HIV. The course of treatment would be as described for the blocker.

In one embodiment, the recombinant alphavirus vector carries a gene specifying a product which is not in itself toxic but, when processed or modified by a protein such as a protease specific to a viral or other pathogen, is converted into a toxic form. For example, the recombinant alphavirus could carry a gene encoding a proprotein for ricin A chain, which becomes toxic upon processing by the HIV protease. More specifically, a synthetic inactive proprotein form of the toxin ricin or diphtheria A chains could be cleaved to the active form by arranging for the HIV virally encoded protease to recognize and cleave off an appropriate "pro" element.

In another embodiment, the alphavirus construct may express a "reporting product" on the surface of the target cells in response to the presence of an identifying agent in the cells (such as expression of a viral gene). This surface protein can be recognized by a cytotoxic agent, such as antibodies for the reporting protein, or by cytotoxic T cells. In a similar manner, such a system can be used as a detection system (see below) to simply identify those cells having a particular gene which expresses an identifying protein.

Similarly, in another embodiment, a surface protein could be expressed which would itself be therapeutically beneficial. In the particular case of HIV, expression of the human CD4 protein specifically in HIV-infected cells may be beneficial in two ways:

1. Binding of CD4 to HIV env intracellularly could inhibit the formation of viable viral particles, much as soluble CD4 has been shown to do for free virus, but without the problem of systematic clearance and possible immunogenicity, since the protein will remain mem-



brane bound and is structurally identical to endogenous CD4 (to which the patient should be immunologically tolerant).

2. Since the CD4/HIV env complex has been implicated as a cause of cell death, additional expression of CD4 (in the presence of excess HIV-env present in HIV-infected cells) leads to more rapid cell death and thus inhibits viral dissemination. This may be particularly applicable to monocytes and macrophages, which act as a reservoir for virus production as a result of their relative refractility to HIV-induced cytotoxicity (which, in turn, is apparently due to the relative lack of CD4 on their cell surfaces).

In another embodiment, the alphavirus vector codes for a ribozyme which will cleave and inactivate RNA molecules essential for viability of the vector infected cell. By making ribozyme production dependent on a specific RNA sequence corresponding to the pathogenic state, such as HIV tat, toxicity is specific to the pathogenic state.

#### 5. EXPRESSION OF MARKERS

The above-described technique of expressing a palliative in a cell in response to a specific RNA sequence can also be modified to enable detection of a particular gene in a cell which expresses an identifying protein (for example, a gene carried by a particular virus), and hence enable detection of cells carrying that virus. In addition, this technique enables the detection of viruses (such as HIV) in a clinical sample of cells carrying an identifying protein associated with the virus.

This modification can be accomplished by providing a genome coding for a product, the presence of which can be readily identified (the "marker product"), in an alphavirus vector which responds to the presence of the identifying protein in the infected cells. For example, HIV, when it infects suitable cells, makes tat and rev. The indicator cells can thus be provided with a genome (such as by infection with an appropriate recombinant alphavirus) which codes for a marker gene, such as the alkaline phosphatase gene,  $\beta$ -galactosidase gene, or the luciferase gene which is expressed by the recombinant alphavirus upon activation by the tat and/or rev RNA transcript. In the case of  $\beta$ -galactosidase or alkaline phosphatase, exposing the cells to substrate analogues results in a color or fluorescence change if the sample is positive for HIV. In the case of luciferase, exposing the sample to luciferin will result in luminescence if the sample is positive for HIV. For intracellular enzymes such as  $\beta$ -galactosidase, the viral titre can be measured directly by counting colored or fluorescent cells, or by making cell extracts and performing a suitable assay. For the membrane bound form of alkaline phosphatase, virus titre can also be measured by performing enzyme assays on the cell surface using a fluorescent substrate. For secreted enzymes, such as an engineered form of alkaline phosphatase, small samples of culture supernatant are assayed for activity, allowing continuous monitoring of a single culture over time. Thus, different forms of this marker system can be used for different purposes. These include counting active virus, or sensitively and simply measuring viral spread in a culture and the inhibition of this spread by various drugs.

Further specificity can be incorporated into the preceding system by testing for the presence of the virus either with or without neutralizing antibodies to that virus. For example, in one portion of the clinical sample being tested, neutralizing antibodies to HIV may be present; whereas in another portion there would be no neutralizing antibodies. If the tests were negative in the system where there were antibodies and

positive where there were no antibodies, this would assist in confirming the presence of HIV.

Within an analogous system for an in vitro assay, the presence of a particular gene, such as a viral gene, may be determined in a cell sample. In this case, the cells of the sample are infected with a suitable alphavirus vector which carries the reporter gene which is only expressed in the presence of the appropriate viral RNA transcript. The reporter gene, after entering the sample cells, will express its reporting product (such as  $\beta$ -galactosidase or luciferase) only if the host cell expresses the appropriate viral proteins.

These assays are more rapid and sensitive, since the reporter gene can express a greater amount of reporting product than identifying agent present, which results in an amplification effect.

#### 6. IMMUNE DOWN-REGULATION

As briefly described above, the present invention also provides recombinant alphavirus which carry a vector construct capable of suppressing one or more elements of the immune system in target cells infected with the alphavirus.

Briefly, specific down-regulation of inappropriate or unwanted immune responses, such as in chronic hepatitis or in transplants of heterologous tissue such as bone marrow, can be engineered using immune-suppressive viral gene products which suppress surface expression of transplantation (MHC) antigen. Group C adenoviruses Ad2 and Ad5 possess a 19 kd glycoprotein (gp 19) encoded in the E3 region of the virus. This gp 19 molecule binds to class I MHC molecules in the endoplasmic reticulum of cells, and prevents terminal glycosylation and translocation of class I MHC to the cell surface. For example, prior to bone marrow transplantation, donor bone marrow cells may be infected with gp 19-encoding vector constructs which, upon expression of the gp 19, inhibit the surface expression of MHC class I transplantation antigens. These donor cells may be transplanted with low risk of graft rejection and may require a minimal immunosuppressive regimen for the transplant patient. This may allow an acceptable donor-recipient chimeric state to exist with fewer complications. Similar treatments may be used to treat the range of so-called autoimmune diseases, including lupus erythematosus, multiple sclerosis, rheumatoid arthritis or chronic hepatitis B infection.

An alternative method involves the use of anti-sense message, ribozyme, or other specific gene expression inhibitor specific for T cell clones which are autoreactive in nature. These block the expression of the T cell receptor of particular unwanted clones responsible for an autoimmune response. The anti-sense, ribozyme, or other gene may be introduced using the viral vector delivery system.

#### 7. REPLACEMENT OR AUGMENTATION GENE THERAPY

One further aspect of the present invention relates to transforming cells of an animal with recombinant alphavirus vectors which serve as gene transfer vehicles to supply genetic sequences capable of expressing a therapeutic protein. Within one embodiment of the present invention, the viral vector construct is designed to express a therapeutic protein capable of preventing, inhibiting, stabilizing or reversing an inherited or noninherited genetic defect in metabolism, immune regulation, hormonal regulation, enzymatic or membrane associated structural function. This embodiment also describes the viral vector capable of transducing individual cells, whereby the therapeutic protein is able to be expressed systemically or locally from a specific cell or tissue, whereby the therapeutic protein is capable of (a) the replacement of an absent or defective cellular protein



or enzyme, or (b) supplement production of a defective or low expressed cellular protein or enzyme. Such diseases may include cystic fibrosis, Parkinson's disease, hypercholesterolemia, adenosine deaminase deficiency,  $\beta$ -globin disorders, Hemophilia A & B, Gaucher's disease, diabetes and leukemia.

As an example of the present invention, a recombinant alphavirus viral vector can be used to treat Gaucher disease. Briefly, Gaucher disease is a genetic disorder that is characterized by the deficiency of the enzyme glucocerebrosidase. This type of therapy is an example of a single gene replacement therapy by providing a functional cellular enzyme. This enzyme deficiency leads to the accumulation of glucocerebroside in the lysosomes of all cells in the body. However, the disease phenotype is manifested only in the macrophages, except in the very rare neuronopathic forms of the disease. The disease usually leads to enlargement of the liver and spleen and lesions in the bones. (For a review, see *Science* 256:794, 1992, and *The Metabolic Basis of Inherited Disease*, 6th ed., Scriver et al., vol. 2, p. 1677).

#### 8. LYMPHOKINES AND LYMPHOKINE RECEPTORS

As noted above, the present invention provides alphavirus particles which can, among other functions, direct the expression of one or more cytokines or cytokine receptors.

Briefly, in addition to their role as cancer therapeutics, cytokines can have negative effects resulting in certain pathological conditions. For example, most resting T-cells, B cells, large granular lymphocytes and monocytes do not express IL-2R (receptor). In contrast to the lack of IL-2R expression on normal resting cells, IL-2R is expressed by abnormal cells in patients with certain leukemias (AITL, Hairy-cell, Hodgkins, acute and chronic granulocytic), autoimmune diseases, and is associated with allograft rejection. Interestingly, in most of these patients the serum concentration of a soluble form of IL-2R is elevated. Therefore, with certain embodiments of the invention therapy may be effected by increasing the serum concentration of the soluble form of the cytokine receptor. For example, in the case of IL-2R, an alphavirus vector can be engineered to produce both soluble IL-2R and IL-2R, creating a high affinity soluble receptor. In this configuration, serum IL-2 levels would decrease, inhibiting the paracrine loop.

This same strategy may also be effective against autoimmune diseases. In particular, because some autoimmune diseases (e.g., Rheumatoid arthritis, SLE) are also associated with abnormal expression of IL-2, blocking the action of IL-2 by increasing the serum level of receptor may also be utilized in order to treat such autoimmune diseases.

In other cases inhibiting the levels of IL-1 may be beneficial. Briefly, IL-1 consists of two polypeptides, IL-1 and IL-1 $\beta$ , each of which has pleiotropic effects. IL-1 is primarily synthesized by mononuclear phagocytes, in response to stimulation by microbial products or inflammation. There is a naturally occurring antagonist of the IL-1R, referred to as the IL-1 Receptor antagonist ("IL-1Ra"). This IL-1R antagonist has the same molecular size as mature IL-1 and is structurally related to it. However, binding of IL-1Ra to the IL-1R does not initiate any receptor signaling. Thus, this molecule has a different mechanism of action than a soluble receptor, which complexes with the cytokine and thus prevents interaction with the receptor. IL-1 does not seem to play an important role in normal homeostasis. In animals, antibodies to IL-1 receptors reduce inflammation and anorexia due to endotoxins and other inflammation inducing agents.

In the case of septic shock, IL-1 induces secondary compounds which are potent vasodilators. In animals, exog-

enously supplied IL-1 decreases mean arterial pressure and induces leukopenia. Neutralizing antibody to IL-1 reduced endotoxin-induced fever in animals. In a study of patients with septic shock who were treated with a constant infusion of IL-1R for three days, the 28 day mortality was 16% compared to 44% in patients who received placebo infusions.

In the case of autoimmune disease, reducing the activity of IL-1 reduces inflammation. Similarly, blocking the activity of IL-1 with recombinant receptors can result in increased allograft survival in animals, again presumably by decreasing inflammation.

These diseases provide further examples where alphavirus vectors may be engineered to produce a soluble receptor or more specifically the IL-1Ra molecule. For example, in patients undergoing septic shock, a single injection of IL-1Ra producing vector particles could replace the current approach requiring a constant infusion of recombinant IL-1R.

Cytokine responses, or more specifically, incorrect cytokine responses may also be involved in the failure to control or resolve infectious diseases. Perhaps the best studied example is non-healing forms of leishmaniasis in mice and humans which have strong, but counterproductive  $T_H2$ -dominated responses. Similarly, lepromatous leprosy is associated with a dominant, but inappropriate  $T_H2$  response. In these conditions, alphavirus-based gene therapy may be useful for increasing circulating levels of IFN gamma, as opposed to the site-directed approach proposed for solid tumor therapy. IFN gamma is produced by  $T_H1$  T-cells, and functions as a negative regulator of  $T_H2$  subtype proliferation. IFN gamma also antagonizes many of the IL-4 mediated effects on B-cells, including isotype switching to IgE.

IgE, mast cells and eosinophils are involved in mediating allergic reaction. IL-4 acts on differentiating T-cells to stimulate  $T_H2$  development, while inhibiting  $T_H1$  responses. Thus, alphavirus-based gene therapy may also be accomplished in conjunction with traditional allergy therapeutics. One possibility is to deliver alphavirus-IL4R with small amounts of the offending allergen (i.e., traditional allergy shots). Soluble IL-4R would prevent the activity of IL-4, and thus prevent the induction of a strong  $T_H2$  response.

#### 9. SUICIDE VECTOR

One further aspect of the present invention relates to the expression of alphavirus suicide vectors to limit the spread of wild-type alphavirus in the packaging/producer cell lines. Briefly, within one embodiment the alphavirus suicide vector would be comprised of an antisense or ribozyme sequence, specific for the wild-type alphavirus sequence generated from an RNA recombination event between the 3' sequences of the junction region of the vector, and the 5' alphavirus structural sequences of the packaging cell line expression vector. The antisense or ribozyme molecule would only be thermostable in the presence of the specific recombination sequence and would not have any other effect in the alphavirus packaging/producer cell line. Alternatively, a toxic molecule (such as those disclosed below), may also be expressed in the context of a vector that would only express in the presence of wild-type alphavirus.

#### 10. ALPHAVIRUS VECTORS TO PREVENT THE SPREAD OF METASTATIC TUMORS

One further aspect of the present invention relates to the use of alphavirus vectors for inhibiting or reducing the invasiveness of malignant neoplasms. Briefly, the extent of malignancy typically relates to vascularization of the tumor. One cause for tumor vascularization is the production of

soluble tumor angiogenesis factors (TAF) (Pawletz et al., *Crit. Rev. Oncol. Hematol.* 9:197, 1989) expressed by some tumors. Within one aspect of the present invention, tumor vascularization may be slowed by using alphavirus vectors to express antisense or ribozyme RNA molecules specific for TAF. Alternatively, anti-angiogenesis factors (Moses et al., *Science* 248:1408, 1990; Shapiro et al., *PNAS* 84:2238, 1987) may be expressed either alone or in combination with the above-described ribozymes or antisense sequences in order to slow or inhibit tumor vascularization. Alternatively, alphavirus vectors can also be used to express an antibody specific for the TAF receptors on surrounding tissues.

#### 11. ADMINISTRATION OF ALPHAVIRUS PARTICLES

Within other aspects of the present invention, methods are provided for administering recombinant alphavirus vectors or particles. Briefly, the final mode of viral vector administration usually relies on the specific therapeutic application, the best mode of increasing vector potency, and the most convenient route of administration. Generally, this embodiment includes recombinant alphavirus vectors which can be designed to be delivered by, for example, (1) direct injection into the blood stream; (2) direct injection into a specific tissue or tumor; (3) oral administration; (4) nasal inhalation; (5) direct application to mucosal tissues; or (6) ex vivo administration of transduced autologous cells into the animal. Thus the therapeutic alphavirus vector can be administered in such a fashion such that the vector can (a) transduce a normal healthy cell and transform the cell into a producer of a therapeutic protein or agent which is secreted systemically or locally, (b) transform an abnormal or defective cell, transforming the cell into a normal functioning phenotype, (c) transform an abnormal cell so that it is destroyed, and/or (d) transduce cells to manipulate the immune response.

#### I. MODULATION OF TRANSCRIPTION FACTOR ACTIVITY

In yet another embodiment, alphavirus vectors may be utilized in order to regulate the growth control activity of transcription factors in the infected cell. Briefly, transcription factors directly influence the pattern of gene expression through sequence-specific trans-activation or repression (Karin, *New Biologist* 21:126-131, 1990). Thus, it is not surprising that mutated transcription factors represent a family of oncogenes. Alphavirus gene transfer therapy can be used, for example, to return control to tumor cells whose unregulated growth is activated by oncogenic transcription factors, and proteins which promote or inhibit the binding cooperatively in the formation of homo- and heterodimer trans-activating or repressing transcription factor complexes.

One method for reversing cell proliferation would be to inhibit the trans-activating potential of the c-myc/Max heterodimer transcription factor complex. Briefly, the nuclear oncogene c-myc is expressed by proliferating cells and can be activated by several distinct mechanisms, including retroviral insertion, amplification, and chromosomal translocation. The Max protein is expressed in quiescent cells and, independently of c-myc, either alone or in conjunction with an unidentified factor, functions to repress expression of the same genes activated by the myc/Max heterodimer (Cole, *Cell* 65:715-716, 1991).

Inhibition of c-myc or c-myc/Max proliferation of tumor cells may be accomplished by the overexpression of Max in target cells controlled by alphavirus vectors. The Max protein is only 160 amino acids (corresponding to 480 nucleotide RNA length) and is easily incorporated into an

alphavirus vector either independently, or in combination with other genes and/or antisense/ribozyme moieties targeted to factors which release growth control of the cell.

Modulation of homo/hetero-complex association is another approach to control transcription factor activated gene expression. For example, transport from the cytoplasm to the nucleus of the trans-activating transcription factor NF-B is prevented while in a heterodimer complex with the inhibitor protein IB. Upon induction by a variety of agents, including certain cytokines, IB becomes phosphorylated and NF-B is released and transported to the nucleus, where it can exert its sequence-specific trans-activating function (Baeuerle and Baltimore, *Science* 242:540-546, 1988). The dissociation of the NF-B/IB complex can be prevented by masking with an antibody the phosphorylation site of IB. This approach would effectively inhibit the trans-activation activity of the NF-IB transcription factor by preventing its transport to the nucleus. Expression of the IB phosphorylation site specific antibody or protein in target cells may be accomplished with an alphavirus gene transfer vector. An approach similar to the one described here could be used to prevent the formation of the trans-activating transcription heterodimer factor AP-1 (Turner and Tijan, *Science* 243:1689-1694, 1989), by inhibiting the association between the jun and fos proteins.

#### J. PHARMACEUTICAL COMPOSITIONS

As noted above, the present invention also provides pharmaceutical compositions comprising a recombinant Sindbis particle or virus, or Sindbis vector construct, in combination with a pharmaceutically acceptable carrier, diluent, or recipient.

Briefly, infectious recombinant virus (also referred to above as particles) may be preserved either in crude or purified forms. In order to produce virus in a crude form, virus-producing cells may first be cultivated in a bioreactor, wherein viral particles are released from the cells into the culture media. Virus may then be preserved in crude form by first adding a sufficient amount of a formulation buffer to the culture media containing the recombinant virus to form an aqueous suspension. Within certain preferred embodiments, the formulation buffer is an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. The aqueous solution may also contain one or more amino acids.

The recombinant virus can also be preserved in a purified form. More specifically, prior to the addition of the formulation buffer, the crude recombinant virus described above may be clarified by passing it through a filter and then concentrated, such as by a cross flow concentrating system (Filtron Technology Corp., Northborough, Mass.). Within one embodiment, DNase is added to the concentrate to digest exogenous DNA. The digest is then diafiltrated in order to remove excess media components and to establish the recombinant virus in a more desirable buffered solution. The diafiltrate is then passed over a Sephadex S-500 gel column and a purified recombinant virus is eluted. A sufficient amount of formulation buffer is then added to this eluate in order to reach a desired final concentration of the constituents and to minimally dilute the recombinant virus. The aqueous suspension may then be stored, preferably at -70° C., or immediately dried. As above, the formulation buffer may be an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. The aqueous solution may also contain one or more amino acids.

Crude recombinant virus may also be purified by ion exchange column chromatography. Briefly, crude recombi-

nant virus may be clarified by first passing it through a filter, followed by loading the filtrate onto a column containing a highly sulfonated cellulose matrix. The recombinant virus may then be eluted from the column in purified form by using a high salt buffer, and the high salt buffer exchanged for a more desirable buffer by passing the eluate over a molecular exclusion column. A sufficient amount of formulation buffer is then added, as discussed above, to the purified recombinant virus and the aqueous suspension is either dried immediately or stored, preferably at  $-70^{\circ}\text{C}$ .

The aqueous suspension in crude or purified form can be dried by lyophilization or evaporation at ambient temperature. Briefly, lyophilization involves the steps of cooling the aqueous suspension below the gas transition temperature or below the eutectic point temperature of the aqueous suspension, and removing water from the cooled suspension by sublimation to form a lyophilized virus. Within one embodiment, aliquots of the formulated recombinant virus are placed into an Edwards Refrigerated Chamber (3 shelf RC3S unit) attached to a freeze dryer (Supermodulyo 12K). A multistep freeze drying procedure as described by Phillips et al. (*Cryobiology* 18:414, 1981) is used to lyophilize the formulated recombinant virus, preferably from a temperature of  $-40^{\circ}\text{C}$ . to  $-45^{\circ}\text{C}$ . The resulting composition contains less than 10% water by weight of the lyophilized virus. Once lyophilized, the recombinant virus is stable and may be stored at  $-20^{\circ}\text{C}$ . to  $25^{\circ}\text{C}$ ., as discussed in more detail below.

Within the evaporative method, water is removed from the aqueous suspension at ambient temperature by evaporation. Within one embodiment, water is removed through spray-drying (EP 520,748). Within the spray-drying process, the aqueous suspension is delivered into a flow of preheated gas, usually air, whereupon water rapidly evaporates from droplets of the suspension. Spray-drying apparatus are available from a number of manufacturers (e.g., Drytec, Ltd., Tonbridge, England; Lab-Plant, Ltd., Huddersfield, England). Once dehydrated, the recombinant virus is stable and may be stored at  $-20^{\circ}\text{C}$ . to  $25^{\circ}\text{C}$ . Within the methods described herein, the resulting moisture content of the dried or lyophilized virus may be determined through use of a Karl-Fischer apparatus (EM Science Aquastar V1B volumetric titrator, Cherry Hill, N.J.), or through a gravimetric method.

The aqueous solutions used for formulation, as previously described, are preferably composed of a saccharide, high molecular weight structural additive, a buffering component, and water. The solution may also include one or more amino acids. The combination of these components act to preserve the activity of the recombinant virus upon freezing and lyophilization or drying through evaporation. Although a preferred saccharide is lactose, other saccharides may be used, such as sucrose, mannitol, glucose, trehalose, inositol, fructose, maltose or galactose. In addition, combinations of saccharides can be used, for example, lactose and mannitol, or sucrose and mannitol. A particularly preferred concentration of lactose is 3%-4% by weight. Preferably, the concentration of the saccharide ranges from 1% to 12% by weight.

The high molecular weight structural additive aids in preventing viral aggregation during freezing and provides structural support in the lyophilized or dried state. Within the context of the present invention, structural additives are considered to be of "high molecular weight" if they are greater than 5000 m.w. A preferred high molecular weight structural additive is human serum albumin. However, other substances may also be used, such as hydroxyethyl-

cellulose, hydroxymethyl-cellulose, dextran, cellulose, gelatin, or povidone. A particularly preferred concentration of human serum albumin is 0.1% by weight. Preferably, the concentration of the high molecular weight structural additive ranges from 0.1% to 10% by weight.

The amino acids, if present, function to further preserve viral infectivity upon cooling and thawing of the aqueous suspension. In addition, amino acids function to further preserve viral infectivity during sublimation of the cooled aqueous suspension and while in the lyophilized state. A preferred amino acid is arginine, but other amino acids such as lysine, ornithine, serine, glycine, glutamine, asparagine, glutamic acid or aspartic acid can also be used. A particularly preferred arginine concentration is 0.1% by weight. Preferably, the amino acid concentration ranges from 0.1% to 10% by weight.

The buffering component acts to buffer the solution by maintaining a relatively constant pH. A variety of buffers may be used, depending on the pH range desired, preferably between 7.0 and 7.8. Suitable buffers include phosphate buffer and citrate buffer. A particularly preferred pH of the recombinant virus formulation is 7.4, and a preferred buffer is tromethamine.

In addition, it is preferable that the aqueous solution contain a neutral salt which is used to adjust the final formulated recombinant alphavirus to an appropriate isotonic salt concentration. Suitable neutral salts include sodium chloride, potassium chloride or magnesium chloride. A preferred salt is sodium chloride.

Aqueous solutions containing the desired concentration of the components described above may be prepared as concentrated stock solutions.

It will be evident to those skilled in the art, given the disclosure provided herein, that it may be preferable to utilize certain saccharides within the aqueous solution when the lyophilized virus is intended for storage at room temperature. More specifically, it is preferable to utilize disaccharides, such as lactose or trehalose, particularly for storage at room temperature.

The lyophilized or dehydrated viruses of the subject invention may be reconstituted using a variety of substances, but are preferably reconstituted using water. In certain instances, dilute salt solutions which bring the final formulation to isotonicity may also be used. In addition, it may be advantageous to use aqueous solutions containing components known to enhance the activity of the reconstituted virus. Such components include cytokines, such as IL-2, polycations, such as protamine sulfate, or other components which enhance the transduction efficiency of the reconstituted virus. Lyophilized or dehydrated recombinant virus may be reconstituted with any convenient volume of water or the reconstituting agents noted above that allow substantial, and preferably total solubilization of the lyophilized or dehydrated sample.

The following examples are offered by way of illustration, and not by way of limitation.

## EXAMPLES

### Example 1

#### CLONING OF A SINDBIS GENOMIC LENGTH cDNA

The nature of viruses having an RNA genome of positive polarity is such that, when introduced into a eukaryotic cell which serves as a permissive host, the purified genomic nucleic acid serves as a functional message RNA (mRNA)

molecule for translation of the viral replicase proteins. Therefore, this genomic RNA, purified from the virus, can initiate the same infection cycle that is characteristic of infection by the wild-type virus from which the RNA was purified.

For example, Sindbis virus strain AR339 (ATCC #VR-1248, Taylor et al., *Am. J. Trop. Med. Hyg.* 4:844 1955; isolated from the mosquito *Culex univittatus*) is propagated in baby hamster kidney (BHK-21) cells (ATCC #CCL-10), infected at low multiplicity (0.1 PFU/cell). Alternatively, another HR-derived Sindbis virus strain, obtained from Lee Biomolecular (San Diego, Calif.), also is

of the Apa I recognition sequence facilitates insertion of the PCR amplicon into the plasmid vector (pKS II\*, Stratagene, San Diego, Calif.) polylinker sequence. A five nucleotide 'buffer sequence' is also inserted prior to the Apa I recognition sequence in order to permit efficient digestion. The sequence of the SP6-5' Sindbis forward primer and all of the primer pairs necessary to amplify the entire Sindbis genome are shown below. (Note that "nt" and "nts" as utilized hereinafter refer to "nucleotide" and "nucleotides," respectively). The reference sequence (GenBank accession no. SINCG) is from Strauss et al., *Virology* 133:92-110.

Primer	Location	Seq. ID No.	Sequence	Recognition Sequence (5'→3')
SP6-1A	Apa I/SP6/+ SIN nts. 1-18	4	TATATGGGCCCGATTAGGTGAC ACTATAGATTGACGGCGTAGTAC AC	Apa I
1B	3182-3160	5	CTGGCAACCGGTAAGTACGATAC	Age I
2A	3144-3164	6	ATACTAGCCACGGCCGGTATC	Age I
2B	5905-5885	7	TCCTCTTTCGACGTGTCGAGC	Eco RI
3A	5844-5864	8	ACCTTGGAGCGCAATGTCTTG	Eco RI
7349R	7349-7328	9	CCTTTTCAGGGGATCCGCCAC	Bam HI
7328F	7328-7349	10	GTGGCGGATCCCTGAAAAGG	Bam HI
3B	9385-9366	11	TGGGCCGTGTGTCGTCATG	Bcl I
4A	9336-9356	12	TGGGTCCTCAACTCACCGGAC	Bcl I
10394R	10394-10372	13	CAATTCGACGTACGCCCTCACTC	Bsi WI
10373F	10373-10394	14	GAGTGAGGCGTACGTCGAATTG	Bsi WI
4B	Xba I/dT <sub>25</sub> / 11703-11698	3	TATATTCTAGA(dT) <sub>25</sub> -GAAATG	Xba I

used and propagated by the same methods. Sindbis virions are precipitated from a clarified lysate at 48 hours post-infection, with 10% (w/v) of polyethylene glycol (PEG-8000) at 0° C., as described previously. Sindbis virions which are contained in the PEG pellet are then lysed with 2% SDS, and the polyadenylated mRNA isolated by chromatography utilizing commercially available oligo-dT columns (Invitrogen, San Diego, Calif.).

Two rounds of first strand cDNA synthesis are performed on the polyA selected mRNA, using an oligonucleotide primer with the sequence shown below:

5'-TATATTCTAGA(dT)<sub>25</sub>-GAAATG-3'(SEQ. ID NO.3)

Briefly, this primer contains at its 5' end, a five nucleotide 'buffer sequence' for efficient restriction endonuclease digestion, followed by the Xba I recognition sequence, 25 consecutive dT nucleotides and six nucleotides which are precisely complementary to the extreme Sindbis 3' end. Thus, selection for first round cDNA synthesis occurs at two levels: (1) polyadenylated molecules, a prerequisite for functional mRNA, and (2) selective priming from Sindbis mRNA molecules, in a pool possibly containing multiple mRNA species. Further, the reverse transcription is performed in the presence of 10 mM MeHgOH to mitigate the frequency of artificial stops during reverse transcription.

Primary genomic length Sindbis cDNA is then amplified by PCR in six distinct segments using six pairs of overlapping primers. Briefly, in addition to viral complementary sequences, the Sindbis 5' end forward primer is constructed to contain a 19 nucleotide sequence corresponding to the bacterial SP6 RNA polymerase promoter and the Apa I restriction endonuclease recognition sequence linked to its 5' end. The bacterial SP6 RNA polymerase is poised such that transcription in vitro results in the inclusion of only a single non-viral G ribonucleotide linked to the A ribonucleotide, which corresponds to the authentic Sindbis 5' end. Inclusion

PCR amplification of Sindbis cDNA with the six primer sets shown above is performed in separate reactions, using the THERMALASE™ thermostable DNA polymerase (Amresco Inc., Solon, Ohio) and the buffer containing 1.5 mM MgCl<sub>2</sub>, provided by the supplier. Additionally, the reactions contain 5% DMSO, and the HOT START WAX™ beads (Perkin-Elmer), using the PCR amplification protocol shown below:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	3.5	
72	10	10

Following amplification, the six reaction products are inserted first into the pCR II vector (Invitrogen), then using the appropriate enzymes shown above, are inserted, stepwise, into the pKS II\* (Stratagene) vector, between the Apa I and Xba I sites. This clone is designated as pVGSP6GEN.

The Sindbis genomic cDNA clone pVGSP6GEN is linearized by digestion with Xba I, which cuts pVGSP6GEN once, immediately adjacent and downstream of the 25 nucleotide long poly dA:dT stretch. The linearized pVGSP6GEN clone is purified with GENECLEAN™ (BIO 101, La Jolla, Calif.), and adjusted to a concentration of 0.5 mg/ml. Transcription of the linearized pVGSP6GEN clone is performed in vitro at 40° C. for 90 minutes according to the following reaction conditions: 2 ul DNA/4.25 ul H<sub>2</sub>O; 10 ul 2.5 mM NTPs (UTP, ATP, GTP, CTP); 1.25 ul 20 mM Me<sup>7</sup>G(5')ppp(5')G cap analogue; 1.25 ul 100 mM DTT; 5 ul 5X transcription buffer (Promega, Madison, Wis.); 0.5 ul RNasin (Promega); 0.25 ul 10 mg/ml bovine serum albumin; and 0.5 ul SP6 RNA polymerase (Promega). The in vitro

transcription reaction products can be digested with DNase I (Promega) and are purified by sequential phenol/CHCl<sub>3</sub> and ether extraction, followed by ethanol precipitation, or alternatively, can be used directly for transfection. The in vitro transcription reaction products or purified RNA are complexed with a commercial cationic lipid compound (for example, LIPOFECTIN™, GIBCO-BRL, Gaithersburg, Md.), and applied to BHK-21 cells maintained in a 60 mm petri dish at 75% confluency. The transfected cells are incubated at 30° C. After 94 hours post-transfection, extensive cytopathologic effects (CPE) are observed. No obvious CPE is observed in plates not receiving RNA transcribed from the Sindbis cDNA clone. Further, 1 ml of supernatant taken from transfected cells, added to fresh monolayers of BHK-21 cells, and incubated at 30° C. or 37° C. results in obvious CPE within 18 hours. This demonstrates that the Sindbis cDNA clone pVGSP6GEN is indeed infectious.

Sequence analysis of pVGSP6GEN, shown in Table 1, reveals multiple sequence differences between the Sindbis genomic clone described herein, and the viral clone sequence provided in Genbank (GenBank Accession No. SINCG). Many sequence differences result in the substitution of non-conservative amino acids changes in the Sindbis proteins. To address which sequence changes are unique to the virus strain used for cloning, as described herein, or are a result of cloning artifact, virion RNA is amplified by RT-PCR as described above, and sequence relating to the nucleotides in question is determined by direct sequencing of the RT-PCR amplicon product, using a commercially available kit (Promega, Madison Wis.), and compared to the corresponding pVGSP6GEN sequence. The results of this study are given in Table 2. Briefly, three non-conservative amino acid changes, Gly→Glu, Asp→Gly, and Tyr→Cys, which are a result of cloning artifact are observed respectively at viral nucleotides 2245, 6193, and 6730. These nucleotide changes resulting in non-conservative amino acid changes all map to the viral non-structural protein (NSP) genes, nt 2245 to NSP 2, and nts 6193 and 6730 to NSP 4.

Repair of the NSP 2 and NSP 4 genes is accomplished by RT-PCR, as described above, using virion RNA from a 5 times plaque purified stock. The SP6-1A/1B primer pair described above is used to repair the nt 2245 change. The RT-PCR amplicon product is digested with Eco 47III and Bgl II, and the 882 bp fragment is purified by 1% agarose/TBE gel electrophoresis, and exchanged into the corresponding region of the pVGSP6GEN clone, prepared by digestion with Eco 47III and Bgl II, and treatment with CIAP. The 3A/7349R primer pair described above is used to repair the nt 6193 and nt 6730 changes. The RT-PCR amplicon product is digested with Eco RI and Hpa I, and the 1,050 bp fragment is purified by 1% agarose/TBE gel electrophoresis, and exchanged into the corresponding region of the pVGSP6GEN clone. This clone is designated pVGSP6GENrep. Transfection of BHK cells with in vitro transcribed RNA from pVGSP6GENrep DNA, linearized by digestion with Xba I as described above, results in extensive CPE within 18 hours post-transfection.

TABLE 1

SINDBIS GENOMIC CLONE DIFFERENCES BETWEEN pVGSP6GEN AND GENBANK				
SIN nt #	Change	Codon Change	Location in Codon	amino acid change
Noncoding Region:				
45	T→C	N.A.	N.A.	N.A.
Non-structural Proteins:				
353	C→T	UAU→UAC	3'	Tyr→Tyr
1095	A→C	AUA→CUA	1'	Ile→Leu
1412	T→C	UUU→UUC	3'	Phe→Phe
2032	A→G	GAG→GGG	2'	Glu→Gly
2245	G→A	GGG→GAG	2'	Gly→Glu
2258	A→C	UCA→UCC	3'	Ser→Ser
2873	A→G	CAA→CAG	3'	Gln→Gln
2992	C→T	CCC→CUC	2'	Pro→Leu
3544	T→C	GUC→GCC	2	Val→Leu
3579	A→G	AAA→GAA	1'	Leu→Glu
3822	A→G	ACC→GCC	1'	Thr→Ala
3851	T→C	CUU→CUC	3'	Leu→Leu
5351	A→T	CAA→CAU	3'	Gln→His
5466	G→A	GGU→AGU	1'	Gly→Ser
5495	T→C	AUU→AUC	3'	Ile→Ile
5543	A→T	ACA→ACU	3'	Thr→Thr
5614	T→C	GUA→GCA	2'	Val→Ala
6193	A→G	GAC→GGC	2'	Asp→Gly
6564	G→A	GCA→ACA	1'	Ala→Thr
6730	A→G	UAC→UGC	2'	Tyr→Cys
Structural Proteins:				
8637	A→G	AUU→GUU	1'	Ile→Val
8698	T→A	GUA→GAA	2'	Val→Glu
9108	AAG del	AAG→del	1'-3'	Glu→del
9144	A→G	AGA→GGA	1'	Arg→Gly
9420	A→G	AGU→GGU	1'	Ser→Gly
9983	T→G	GCU→GCG	3'	Ala→Ala
10469	T→A	AUU→AUA	3'	Ile→Ile
10664	T→C	UUU→UUC	3'	Phe→Phe
10773	T→G	UCA→GCA	1'	Ser→Ala

TABLE 2

SINDBIS GENOMIC CLONE ARTIFACT ANALYSIS			
SIN nt #	Amino Acid change	pVGSP6GEN Unique	Cloning Artifact
Nonstructural Proteins:			
2032	Glu→Gly	+	
2245	Gly→Glu		+
2258	Ser→Ser	+	
2873	Gln→Gln	+	
2992	Pro→Leu	+	
3544	Val→Leu		+
3579	Leu→Glu	+	
3822	Thr→Ala		+
3851	Leu→Leu		+
5351	Gln→His	+	
5466	Gly→Ser		+
5495	Ile→Ile		+
5543	Thr→Thr		+
6193	Asp→Gly		+
6730	Tyr→Cys		+
Structural Proteins:			
8637	Ile→Val	+	
8698	Val→Glu	+	
9108	Glu→del	+	
9144	Arg→Gly	+	

\*Mixture: Both Genbank and pVGSP6GEN Sindbis strains present at this nucleotide.

## 55

## Example 2

**GENERATION OF DNA VECTORS WHICH  
INITIATE ALPHAVIRUS INFECTION:  
EUKARYOTIC LAYERED VECTOR INITIATION  
SYSTEMS**

As noted above, the present invention provides eukaryotic layered vector initiation systems which generally comprise a promoter which is capable of initiating the 5' synthesis of RNA from cDNA, a construct which is capable of autonomous or autocatalytic replication in a cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription termination. Within one embodiment, such constructs may be constructed of the following ordered elements: a 5' eukaryotic promoter capable of initiating the synthesis of viral RNA at the authentic alphavirus 5' end, a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-structural proteins, a viral junction region, a heterologous sequence, an alphavirus RNA polymerase recognition sequence, and a 3' transcription termination/polyadenylation signal sequence. Such alphavirus cDNA expression vectors may also include intervening sequences (introns), which are spliced from the pre-RNA in the nucleus prior to transport to the cytoplasm, and which may improve the overall efficiency of the system, in terms of molecules of functional mRNA transported to the cytoplasm/nuclear DNA template. The intron splicing signals are located, for example, between Sindbis and heterologous gene regions as described in Example 3.

Construction of a eukaryotic layered vector initiation system utilizing the Sindbis clone pVGSP6GENrep and mammalian RNA polymerase II promoters is accomplished as follows. Briefly, plasmid pVGSP6GENrep is digested with Bgl II and Xba I, and the reaction products are electrophoresed on a 0.8% agarose/TBE gel. The resulting 9,438 bp fragment is excised, purified with GENECLAN™, and ligated into the 4,475 bp vector fragment resulting from treatment of pCDNA3 (Invitrogen) with Bgl II, Xba I, and CIAP. This construction is designated as pcDNASINbgl/xba.

The U3 region of the long terminal repeat (LTR) from Moloney murine leukemia virus (Mo-MLV) is positioned at the 5' viral end such that the first transcribed nucleotide is a single G residue, which is capped in vivo, followed by the Sindbis 5' end. Juxtaposition of the Mo-MLV LTR and the Sindbis 5' end is accomplished by overlapping PCR as described below. Amplification of the Mo-MLV LTR in the first primary PCR reaction is accomplished in a reaction containing the BAG vector (Price et al., *PNAS* 84:156-160, 1987) and the following primer pair:  
Forward primer: BAGBgl2F1 (buffer sequence/Bgl II recognition sequence/Mo-MLV LTR nts 1-22):

5'-TATATAGATCTAATGAAAGACCCACCTGTAGG  
(SEQ. ID NO. 15)

Reverse primer: BAGwt441R2 (SIN nts 5-1/Mo-MLV LTR nts 441-406):

5'-TCAATCCCGAGTGAGGGGTGTGGGCTCTTTTATTGAGC  
(SEQ. ID NO. 16)

PCR amplification of the Mo-MLV LTR with the primer pair shown above is performed using the THERMALASE™

## 56

thermostable DNA polymerase and the buffer containing 1.5 mM MgCl<sub>2</sub>, provided by the supplier. Additionally, the reaction contains 5% DMSO, and the HOT START WAX™ beads, using the PCR amplification protocol shown below:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	0.5	
72	10	1

Amplification of the Sindbis 5' end in the second primary PCR reaction is accomplished reaction containing the pVGSP6GENrep clone and the following primer

Forward primer: (Mo-MLV LTR nts 421-441/SIN nts 1-16):

5'-CCACAACCCCTCACTCGGGGATTGACGGCGTAGTAC  
(SEQ. ID NO. 17)

Reverse primer: (SIN nts 3182-3160):

5'-CTGGCAACCGGTAAGTACGATAC  
(SEQ. ID NO. 18)

PCR amplification of the Mo-MLV LTR is accomplished with the primer pair and amplification reaction conditions described above, utilizing the PCR amplification protocol shown below:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	3.0	
72	10	1

The 457 bp and 3202 bp products from the primary PCR reactions are purified with GENECLAN™, and combined in a secondary PCR reaction with the following primer pair:

Forward primer: BAGBgl2F1 (buffer sequence/Bgl II recognition sequence/Mo-MLV LTR nts 1-22):

5'-TATATAGATCTAATGAAAGACCCACCTGTAGG  
(SEQ. ID NO. 15)

Reverse primer: (SIN nts 2300-2278):

5'-GGTAACAAGATCTCGTGCCGTG  
(SEQ. ID NO. 19)

PCR amplification of the primer PCR amplicon products is accomplished utilizing the primer pair and amplification reaction conditions shown above, and using the following PCR amplification protocol:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	3.0	
72	10	1

The 25 3' terminal bases of the first primary PCR amplicon product overlaps with the 25 5' terminal bases of the second primary PCR amplicon product; the resultant 2,752 bp overlapping secondary PCR amplicon product is purified by 0.8% agarose/TBE electrophoresis, digested with Bgl II, and the 2,734 bp product is ligated into pcDNASINbgl/xba treated with Bgl H and CIAP. The resulting construction is 16,656 bps and is designated pVGELVIS. The sequence of

5'-TATATATATATGCGGCCGCTTCTTTTATTAATCAACAAAATTTGTTTTTAA  
(SEQ. ID NO. 20)

Reverse Primer: SINSac11700R (buffer sequence/Sac I site  
dT25/SIN nts 11700-11692):

5'-TATATGAGCTCTTTTTTTTTTTTTTTTTTTTTTTTGAAATGTTAAAA  
(SEQ. ID NO. 21)

pVGELVIS is given in FIG. 3 (SEQ.ID NO. 1). Sindbis nucleotides are contained within bases 1-11,700 of the sequence.

pVGELVIS plasmid DNA is complexed with LIPO-FECTAMINE™ (GIBCO-BRL, Gaithersburg, Md.) according to the conditions suggested by the supplier (ca. 5 ug DNA/8 ug lipid reagent) and added to 35 mm wells containing BHK-21 cells at approximately 75% confluency. Cytopathic effects (CPE), characteristic of wild type Sindbis virus infection are observed within 48 hours post-infection. Addition of 1 ml of transfection supernatant to fresh BHK-21 monolayers results in CPE within 16 hrs. This data demonstrates the correct juxtaposition of viral cDNA and RNA polymerase II expression cassette signals in the pVGELVIS construct, resulting in the de novo initiation of an RNA virus from a DNA expression module.

In order to determine the relative efficiency of the pVGELVIS plasmid DNA to initiate infection characteristic of wild type Sindbis virus after transfection into BHK cells, an infectious centers assay is performed. Briefly, 5 ug of pVGELVIS plasmid DNA is transfected into BHK-21 cells in 35 mm wells as described above, and at 1.5 hours post transfection the cells are trypsinized and serially diluted 10,000-fold, over 10-fold increments, into 5x10<sup>5</sup> untreated BHK cells. This transfected and untreated BHK cell mixture is then added to 35 mm wells. The cells are allowed to attach to the plate, and subsequently overlaid with media containing 1.0% Noble Agar. At 48 hrs post transfection, plaques due to cell lysis (as a result of Sindbis virus replication) are visualized either directly or after overlaying with a second layer containing Neutral Red Stain. This experiment reveals that the efficiency of the pVGELVIS plasmid in generating wild type Sindbis virus after transfection onto BHK cells is approximately 1x10<sup>4</sup> PFU/ug of plasmid DNA.

## Example 3

PREPARATION OF RNA AND DNA  
ALPHAVIRUS VECTORS

## A. CONSTRUCTION OF THE SINDBIS BASIC VECTOR

A first step in the construction of the Sindbis Basic Vector is the generation of two plasmid subclones containing separate elements from the viral 5' and 3' ends. These elements may then be utilized in order to subsequently assemble a basic gene transfer vector.

Briefly, the first plasmid subclone is constructed to contain the 40 terminal nucleotides of the viral 3' end and a 25 base pair stretch of consecutive dA:dT nucleotides. In particular, the following oligonucleotide pairs are first synthesized:

Forward Primer: SIN11664F: (buffer sequence/Not I site/SIN nts 11664-11698):

The above oligonucleotides are then mixed together at equal molar concentrations in the presence of 10 mM MgCl<sub>2</sub>, heated to 100° C. for 5 minutes and cooled slowly to room temperature. The partially double-stranded molecule is then filled in using Klenow DNA polymerase and 50 uM dNTPs. The resultant 89 bp molecule is then digested with Not I and Sac I, purified on a 2% NuSieve/1% agarose gel, and ligated into pKS II+ plasmid (Stratagene, La Jolla, Calif.), prepared by digestion with Not I and Sac I and treatment with CIAP, at a 10:1 molar excess of insert:vector ratio. This construction is designated pKSI13' SIN.

The second plasmid subclone is constructed to contain the first 5' 7,643 nucleotides of Sindbis, and a bacteriophage RNA polymerase promoter is positioned at the viral 5' end such that only a single non-viral nucleotide is added to the authentic viral 5' end after in vitro transcription. Briefly, the 3' end of this clone is derived by a standard three temperature PCR amplification with a reverse primer having the sequence shown below.

Reverse Primer: SINXho7643R (buffer sequence/Xho I site/SIN nts 7643-7621):

5'TATATCTCGAGGGTGGTGTGTAGTATTAGTCAG  
(SEQ. ID NO. 22)

The reverse primer maps to viral nucleotides 7643-7621 and is 41 bp downstream from the junction core element 3' end. Additionally, viral nucleotide 7643 is 4 nucleotides upstream from the structural protein gene translation initiation codon. The first five 5' nucleotides in this primer are included to serve as a 'buffer sequence' for the efficient digestion of the PCR amplicon products, and are followed by 6 nucleotides comprising the Xho I recognition sequence.

The forward primer in this reaction is primer 2A (described in Example 1), having the following sequence:

ATACTAGCCACGGCCGGTATC (SEQ. ID NO. 6)

The 4510 bp amplicon product, resulting from the PCR amplification shown above with pVGSP6GENrep plasmid (described in Example 1) as template, is digested with the enzymes Sfi I and Xho I. The resultant 2526 bp fragment is gel purified. Sindbis cDNA clone pVGSP6GENrep is also digested with Apa I and Sfi I, and the resultant 5144 bp fragment which includes the SP6 RNA polymerase promoter at its 5' end is gel purified. The 5144 bp fragment is ligated together with the 2526 bp fragment from above, along with Apa I and the Xho I digested CIAP treated pKS II+ plasmid. A clone is isolated having the Sindbis nucleotides 1-7643 including the RNA polymerase promoter at its 5' end contained in the pKSII+ plasmid vector. This construction is designated pKSII5' SIN.

Assembly of the complete basic vector is accomplished by digesting pKSII5' SIN with Xho I and Sac I, treating with CIAP, and gel purifying of a large 10,533 bp fragment. The 10,533 bp fragment is then ligated together with a 168 bp small fragment resulting from digestion of pKSII3' SIN with Xho I and Sac I. This resultant construction is designated pKSSINBV (also known as SINDBIS basic vector, see FIG. 4).

#### B. CONSTRUCTION OF SINDBIS LUCIFERASE VECTOR

The firefly luciferase reporter gene is inserted into the Sindbis Basic Vector in order to demonstrate the expression of a heterologous gene in cells transfected with RNA that is transcribed in vitro from the Sindbis vector clone, and to demonstrate the overall functionality of the Sindbis basic vector.

Construction of the Sindbis luciferase vector is performed by assembling together components of 3 independent plasmids: pKSII5' SIN, pKSII3' SIN, and pGL2-basic vector. The pGL2-basic vector plasmid (Promega, Madison, Wis.) contains the entire firefly luciferase gene. Briefly, the luciferase gene is first inserted into the pKSII3' SIN plasmid. This is accomplished by digesting pGL2 with Bam HI and Hind III, and gel purifying a 2689 bp containing fragment. This fragment is ligated with a gel purified 3008 bp large fragment resulting from digestion of pKSII3' SIN with Bam HI and Hind III and treatment with CIAP. The resultant construction is designated pKSII3' SIN-luc.

Final assembly of a Sindbis luciferase vector is accomplished by digesting pKSII5' SIN with Xho I and Sac I, treating with CIAP, and gel purifying the large 10,533 bp fragment. The pKS 5' SIN 10,533 bp fragment is ligated together with the 2854 bp small fragment resulting from digestion of pKSII3' SIN-luc with Xho I and Sac I. This construction contains the entire Sindbis nonstructural gene coding region and 3' viral elements necessary for genome replication, as well as the firefly luciferase gene positioned between these two viral 5' and 3' elements. This vector is designated pKSSINBV-luc (also known as SINDBIS-luciferase) and is shown schematically in FIG. 4.

#### C. EXPRESSION OF LUCIFERASE IN TRANSFECTED AND INFECTED BHK-21 CELLS

In order to test the functionality of the Sindbis Basic Vector, the expression of luciferase in cells transfected with RNA transcribed in vitro from Sac I-linearized pKSSINBV-luc, as described in Example 1, is tested.

In addition, a complementary packaging vector, which is deleted of most of the non structural gene region, is constructed by digestion of pVGSP6GENrep with Bsp EI and

re-ligation under dilute conditions. This construction, designated pVGSP6GENd1Bsp (also known as "d1Bsp EI") is deleted of nonstructural gene sequences between bases 422-7,054, and is shown schematically in FIG. 5. Transcription in vitro of Xba I-linearized pVGSP6GENd1Bsp is as described in Example 1. Transfections and co-transfections are performed by complexing in vitro transcription products with LIPOFECTIN™ and applying to BHK-21 cells. The expression of luciferase in transfected cells is tested 18 hours after transfection. Additionally, 1 ml of the transfection supernatant is used to infect a confluent monolayer of BHK-21 cells and the expression of luciferase is tested at 24 hours post-infection.

The results of this experiment shown in FIG. 6, demonstrate clearly abundant reporter gene expression follows transfection of BHK-21 cells with in vitro transcribed RNA from pKSSINBV-luc, and transfer (e.g., packaging) of the expression activity when cells are co-transfected with in vitro transcribed RNA from pVGSP6GENd1Bsp.

#### D. CONSTRUCTION OF ALTERED JUNCTION REGION SINDBIS VECTORS

In order to inactivate the Sindbis viral junction region, nucleotides within the NSP4 carboxy terminus and junction region overlap are changed, and the vector nucleotides corresponding to Sindbis are terminated prior to the subgenomic initiation point at Sindbis nt 7598. This construction is shown schematically in FIG. 7.

Briefly, a fragment is PCR amplified from the pKSSINBV clone under nonstringent reaction cycle conditions utilizing a reverse primer having the following sequence:

TATATGGGCCCTTAAGAOCATCGGAGCGATGCTTATTTCCCC  
(SEQ. ID NO. 23)

The underlined bases in the reverse primer relate to nucleotide changes which can be made in the junction region without affecting the coded amino acid (see below). All of the nucleotide changes are transversions.  
3' end of NSP 4 (viral nts 7580-7597):

TCT	CTA	CGG	TGG	TCC	TAA	(SEQ. ID NO. 24)
ser	leu	arg	trp	ser	stop	(SEQ. ID NO. 25)
G	C	A	T	T		
(resulting nt changes from reverse primer)						

The reverse primer is complementary to Sindbis nts 7597-7566 (except at nucleotides, as shown, where junction region changes were made), and includes at its 5' end the 6 nucleotide Apa I recognition sequence following a 5' terminal TATAT tail 'buffer sequence' for efficient enzyme digestion.

The forward primer in this reaction is primer 2A (described in Example 1), having the following sequence:

5'-ATACTAGCCACGGCCGGTATC  
(SEQ. ID NO. 6)

The 4,464 bp amplicon resulting from a PCR reaction with pKSSINBV template and using the primer pair described above is digested with Sfi I and Apa I and the gel purified 2,480 bp fragment is ligated together with the gel purified 5,142 bp fragment resulting from the digestion of pKSSINBV with Apa I and Sfi I, and with the gel purified 2,961 bp fragment resulting from the digestion of pKSII+ with Apa I and from the treatment with CIAP. This



construction, comprised of Sindbis nucleotides 1–7597, including the changes in the junction region described above, and including the bacterial SP6 promoter attached to Sindbis nt 1 is referred to as pKSS' SIND1JR.

Final construction of the inactivated junction region vector is accomplished by ligation of the 7,622 bp large Sindbis fragment resulting from digestion of pKSS' SIND1JR with Apa I, with the 3,038 bp fragment resulting from digestion of pKSSII3' SIN with Apa I and treatment with CIAP. The positive orientation of the 5' Sindbis element, relative to the 3' Sindbis element, is confirmed by restriction endonuclease analysis. This construction is referred to as pKSSINBVd1JR.

Initiation and synthesis of subgenomic mRNA cannot occur from the pKSSINBVd1JR vector. In order to prove this supposition, comparative RNase protection assays using the pKSSINBV and pKSSINBVd1JR vectors are performed. Briefly, a <sup>32</sup>P-end labeled RNA probe complementary in part to the junction region, including the subgenomic RNA initiation point at viral nt 7,598 is used to hybridize with the viral RNA resulting from the transfection of BHK-21 cells with the pKSSINBV and pKSSINBVd1JR vectors. The RNase protection assay demonstrates that cells transfected with pKSSINBV have two fragments, of genomic and subgenomic specificity, while cells transfected with pKSSINBVd1JR have only a single fragment of genomic specificity. These results prove that the junction region in the pKSSINBVd1JR vector is indeed inactivated.

In order to test translation of genomic RNA from the region corresponding to the subgenomic RNA message, the luciferase reporter gene is inserted into the inactivated junction region vector pKSSINBVd1JR described above. This construction is accomplished by digesting the pKSSINBVd1JR with Xho I and Sac I, treating with CIAP, and gel purifying the resulting 10,197 bp fragment. The pKSSINBVd1JR fragment is ligated together with the 2854 bp small fragment resulting from digestion of pKSSII3' SIN-luc with Xho I and Sac I. This construction contains the entire Sindbis nonstructural gene coding region terminating in an inactivated junction region at Sindbis nt 7597, and 3' viral elements necessary for genome replication; the firefly luciferase gene is placed between these two viral 5' and 3' elements. This vector is known as pKSSINBVd1JR-luc.

The expression of the reporter gene from the pKSSINBVd1JR-luc vector is tested in transfected BHK-21 cells. Translation of functional luciferase protein is determined by the luciferin luminescent assay, using a luminometer for detection. The sensitivity in this assay is  $1 \times 10^{-20}$  moles of luciferase. Given that the molecular weight of luciferase is 62,000 daltons, this limit of detection transforms to 6,020 molecules. Thus, in a typical experiment if only 0.6% of the  $1 \times 10^6$  cells contained in a 60 mm petri dish are transfected with the pKSSINBVd1JR-luc vector, and if these transfected cells express only a single functional molecule of luciferase, the enzymatic activity is detected by the assay used. It is important to demonstrate in this experiment that the junction region of the pKSSINBVd1JR-luc vector is inactivated. This is accomplished by an RNase protection assay, comparing the viral RNA's synthesized in cells transfected with the pKSSINBVd1JR-luc and the pKSSINBV-luc vectors, using the probe described above.

The minimal -19→+5 junction region core oligonucleotide pair, comprised of Sindbis nts 7579–7602, is synthesized in vitro, and flanked with Apa I and Xho I recognition sequences as shown:

oligonucleotide 1:

5'-CATCTCTACGGTGGTCTAAATAGTC  
(SEQ. ID NO. 26)

oligonucleotide 2:

5'-TCGAGACTATTAGGACCAACCGTAGAGATGGGCC  
(SEQ. ID NO. 27)

The oligonucleotides above are mixed together in the presence of 10 mM Mg<sup>2+</sup>, heated to 100° C. for 5 minutes and cooled slowly to room temperature. The annealed oligonucleotides are ligated at a 25:1 molar ratio of insert to the pKSSINBVd1JR vector, prepared accordingly: complete digestion with Xho I, followed by digestion with Apa I under partial conditions, resulting in one Apa I induced cleavage per molecule (of two cleavages possible), gel purification of the 10,655 bp fragment, and treatment with CIAP. This vector containing the entire nonstructural protein coding region which terminates in an inactivated junction region core, attached to a synthetic junction region core and followed by 3' viral elements required for replication, and contained in the pKSSII+ plasmid, is known pKSSINd1JRsrc.

In order to regulate the level of subgenomic mRNA synthesis, further modifications of the tandemly inserted synthetic junction region core in plasmid pKSSINd1JRsrc are performed. These modifications of the junction region core may be accomplished by at least two approaches: nucleotide changes within the junction region core; or extension at the 5' and 3' junction region core termini of flanking Sindbis nucleotides, according to the authentic viral sequence. The minimal junction region core, spanning viral nts 7579–7602 is shown below:

5'-ATCTCTACGGTGGTCTAAATAGT  
(SEQ. ID NO. 2)

By comparing genomic sequence between eight alphaviruses, it has been shown previously that there is sequence diversity within the junction region core. Shown below, for particular junction region locations, is the Sindbis nucleotide followed by the corresponding nucleotide found in other alphaviruses:

Nucleotide Number	Sindbis	Permissive Change
7579	A	C
7580	U	C
7581	C	U
7583	C	G
7589	U	C
7590	G	U
7591	G	A
7592	U	A
7600	A	U or G
7602	U	G or A

Junction region changes at Sindbis nts 7579, 7580, 7581, 7583, 7589, 7590, 7591, 7592, result in potential amino acid coding changes within all 5 codons of the carboxy terminus of NSP 4 which overlap in the junction region. These changes observed in the junction region between alphaviruses at the level of NSP 4 coding potential and at the level

of junction region cis activity may represent either, or both, permissive changes in NSP 4 and the junction region which do not affect functionality, or on the other hand, simply different viruses. In any event, the junction region changes presented herein regard the tandemly inserted junction region core, from which no NSP protein synthesis occurs. Discussed above, translation of the entire NSP region occurs from the pKSSINBVd1JR construct. Junction region changes at Sindbis nts 7600 and 7602 are downstream of the NSP 4 termination codon and upstream of the structural proteins initiation codon.

Locations of nucleotide differences within the junction region core observed between the several alphavirus strains are referred to here as permissive changes. Locations of nucleotides within the junction region core corresponding to conserved sequences between the several alphavirus strains are referred to here as nonpermissive changes.

To decrease the level of subgenomic mRNA initiation from the synthetic junction region core, changes are made separately within nucleotides corresponding to permissive changes, and within nucleotides corresponding to nonpermissive changes. Junction region nucleotides corresponding to permissive changes are given in the table above. Fourteen junction region nucleotides for which no changes are observed among the eight alphaviruses sequenced (Semliki Forest virus, Middleburg virus, Ross River virus, O'Nyong Nyong virus, Eastern Equine Encephalitis virus, Western Equine Encephalitis virus, and Venezuelan Equine Encephalitis virus) are given below:

Nucleotide Number:
7582
7584
7585
7586
7587
7588
7593
7594
7595
7596
7597
7598
7599
7601

Changes within the junction region observed among alphaviruses may reflect a specific interaction between a given alphaviral RNA polymerase and its cognate junction region. Thus, changes among the "permissive" nucleotides may result in as marked a decrease in the subgenomic mRNA synthesis levels as changes among the "nonpermissive" nucleotides of the junction region. On the other hand, these may indeed be sites of permissive change within the junction region core.

The single authentic nonpermissive change within the junction region core is likely Sindbis nt 7598, corresponding to the subgenomic mRNA initiation point. Changes of this nucleotide in the tandemly inserted junction region core of plasmid pKSSINd1JRsirc are not described here.

Substitution of the permissive nucleotides in toto in the synthetic minimal -19→+5 junction region core, is accomplished with the following oligonucleotide pair, synthesized in vitro, and flanked with Apa I and Xho I recognition sequences as shown:

oligonucleotide 1:

5'-CCCTTGTACGGCTAACCTAAAGGAC  
(SEQ. ID NO. 28)

oligonucleotide 2:

5'-TCGAGTCCTTTAGGTTAGCCGTACAAGGGGGCC  
(SEQ. ID NO. 29)

The oligonucleotides above are mixed together in the presence of 10 mM Mg, heated to 100° C. for 5 minutes and cooled slowly to room temperature. The annealed oligonucleotides are ligated at a 25:1 molar ratio of insert to the pKSSINBVd1JR vector, prepared accordingly: complete digestion with Xho I, followed by digestion with Apa I under partial conditions, resulting in one Apa I induced cleavage per molecule (of two cleavages possible), gel purification of the 10,655 bp fragment, and treatment with CIAP. This vector is known as pKSSINd1JRsirPc.

Each of the 13 (nt 7598 not changed) nonpermissive nucleotides in the junction region core are changed individually, using the following rules, resulting in the most drastic transversal substitution:

A→C  
T→G  
G→T  
C→A

For example, nt 7582 is changed from T→G, using the following oligonucleotide pair, synthesized in vitro, and flanked with Apa I and Xho I recognition sequences as shown:

oligonucleotide 1:

5'-CATCGCTACGGTGGTCCTAAATAGTC  
(SEQ. ID NO. 30)

oligonucleotide 2:

5'-TCGAGACTATTAGGACACCGTAGCGATGGGGCC  
(SEQ. ID NO. 31)

(Nucleotides effecting transversion in nonpermissive junction region sites shown in boldface type) The oligonucleotides above are mixed together in the presence of 10 mM Mg<sup>2+</sup>, heated to 100° C. for 5 minutes and cooled slowly to room temperature. The annealed oligonucleotides are ligated at a 25:1 molar ratio of insert to the pKSSINBVd1JR vector, prepared accordingly: complete digestion with Xho I, followed by digestion with Apa I under partial conditions, resulting in one Apa I induced cleavage per molecule (of two cleavages possible), gel purification of the 10,655 bp fragment, and treatment with CIAP. This vector is known pKSSINd1JRsirNP7582.

Using the transversion change rules shown above, changes in each of the 12 remaining nonpermissive sites in the junction region core are made with 12 separate oligonucleotide pairs, flanked with Apa I and Xho I recognition sites, as described above. These vectors are known as:

pKSSINd1JRsirNP7584  
pKSSINd1JRsirNP7585  
pKSSINd1JRsirNP7586

pKSSINd1JRsjrNP7587  
pKSSINd1JRsjrNP7588  
pKSSINd1JRsjrNP7593  
pKSSINd1JRsjrNP7594  
pKSSINd1JRsjrNP7595  
pKSSINd1JRsjrNP7596  
pKSSINd1JRsjrNP7597  
pKSSINd1JRsjrNP7599  
pKSSINd1JRsjrNP7601

In order to test the relative levels of subgenomic mRNA synthesis, the luciferase reporter gene is inserted into the modified tandem junction region vectors. This construction is accomplished by digesting with Xho I and Sac I and treating with CIAP the tandemly inserted synthetic junction region core vectors and gel purifying the resulting approximate 10,200 bp fragment. The treated vector fragment is

should have a lower level of subgenomic mRNA expression, relative to the pKSSINBV construct. Therefore, in certain embodiments, it may be necessary to increase the level of subgenomic mRNA expression observed from the pKSSINd1JRsjrc vector. This may be accomplished by extension at the 5' and 3' synthetic junction region core termini with 11 additional flanking Sindbis nucleotides, according to the authentic viral sequence.

The synthetic oligonucleotide pair shown below is synthesized in vitro, and contains 46 Sindbis nts, including all 24 nts (shown in boldface type) of the minimal junction region core. The Sindbis nts are flanked with the Apa I and Xho I recognition sequences as shown:

oligonucleotide 1:

5'-CGGAAATAAAGCATCTCTACGGTGGTCCTAATAGTCAGCATAGTACC  
(SEQ. ID NO. 32)

then ligated together with the 2854 bp small fragment oligonucleotide 2:

5'-TCGAGGTACTATGCTGACTATTAGGACCAACGTAGAGATGCTTTATTCGGGGCC  
(SEQ. ID NO. 33)

resulting from digestion of pKSI13' SIN-luc with Xho I and Sac I. These constructions contain the entire Sindbis non-structural gene coding region terminating in an inactivated junction region at Sindbis nt 7597, the tandemly inserted synthetic junction region core (modified or unmodified), the firefly luciferase gene, and 3' viral elements necessary for genome replication. The names of these vectors are as follows:

Sindbis-luciferase vector	Tandemly Inserted Junction Region Modification
pKSSINd1JRsjr-luc	not modified
pKSSINd1JRsjrPe-luc	permissive changes
pKSSINd1JRsjrNP7582-luc	nonpermissive change
pKSSINd1JRsjrNP7584-luc	"
pKSSINd1JRsjrNP7585-luc	"
pKSSINd1JRsjrNP7586-luc	"
pKSSINd1JRsjrNP7587-luc	"
pKSSINd1JRsjrNP7588-luc	"
pKSSINd1JRsjrNP7593-luc	"
pKSSINd1JRsjrNP7594-luc	"
pKSSINd1JRsjrNP7595-luc	"
pKSSINd1JRsjrNP7596-luc	"
pKSSINd1JRsjrNP7597-luc	"
pKSSINd1JRsjrNP7599-luc	"
pKSSINd1JRsjrNP7601-luc	"

Assuming that the translation efficiencies are equivalent in all of the luciferase vectors shown immediately above, the relative levels of subgenomic synthesis are determined by comparing the levels of luciferase production at 16 hours post-transfection of BHK-21 cells. The relative levels of subgenomic transcription are determined by comparing luciferase production by the vectors pKSSINBV-luc and pKSSINd1JRsjrc-luc with all of the modified junction region luciferase vectors shown above.

Vectors containing the tandemly inserted synthetic junction region core (pKSSINd1JRsjrc, and derivatives thereof)

The oligonucleotides above are mixed together in the presence of 10 mM Mg, heated to 100° C. for 5 minutes and cooled slowly to room temperature. The annealed oligonucleotides are ligated at a 25:1 molar ratio of insert to the pKSSINBVd1JR vector, prepared accordingly: complete digestion with Xho I, followed by digestion with Apa I under partial conditions, resulting in one Apa I induced cleavage per molecule (of two cleavages possible), gel purification of the 10,655 bp fragment, and treatment with CIAP. This vector containing the entire nonstructural protein coding region which terminates in an inactivated junction region core, attached to an extended synthetic junction region, and followed by 3' viral elements required for replication, and contained in the pKSI1+ plasmid, is known as pKSSINd1JRsexjr.

In order to test the relative levels of subgenomic mRNA synthesis, the luciferase reporter gene is inserted into the extended tandem junction region pKSSINd1JRsexjr vector. This construction is accomplished by digesting the pKSSINd1JRsexjr plasmid with Xho I and Sac I, treating with CIAP, and gel purifying the resulting approximate 10,200 bp fragment. The thus-treated vector fragment is ligated together with the 2854 bp small fragment resulting from digestion of pKSI13' SIN-luc with Xho I and Sac I. This construction contains the entire Sindbis nonstructural gene coding region terminating in an inactivated junction region at Sindbis nt 7597, the tandemly inserted extended synthetic junction region, the firefly luciferase gene, and 3' viral elements necessary for genome replication. The name of this vector is pKSSINd1JRsexjr-luc.

The relative levels of subgenomic transcription are determined by comparing luciferase production by the pKSSINd1JRsexjr-luc vector with the pKSSINBV-luc and pKSSINd1JRsjrc-luc vectors.

#### E. CONSTRUCTION OF PLASMID DNA ALPHAVIRUS EXPRESSION VECTORS

The SINDBIS basic vector and SINDBIS-luciferase constructs described in sections A and B of Example 3, above,

are inserted into the pVGELVIS vector configurations described previously in Example 2 such that expression of the heterologous gene from Sindbis vectors occurs after direct introduction of the plasmid DNA into cells. As described in Example 2, the ability to transfect alphavirus-based vector plasmid DNA directly onto cells resulting in expression levels of heterologous genes typical of transfection of RNA-based alphavirus vectors, without a primary step consisting of in vitro transcription of linearized template vector DNA, enhances greatly the utility and efficiency of certain embodiments of the alphavirus-based expression vector system. FIG. 8 is a schematic representation of one mechanism of expression of heterologous genes from a plasmid DNA alphavirus expression (ELVIS) vectors. Primary transcription in the nucleus and transport of the vector RNA to the cytoplasm leads to the synthesis of alphavirus nonstructural proteins which catalyze the expansion of heterologous gene mRNA via an antigenome intermediate which in turn serves as the template for production of genomic and subgenomic mRNA. The ELVIS vectors may be introduced into the target cells directly by physical means as a DNA molecule, as a complex with various liposome formulations, or as a DNA ligand complex including the alphavirus DNA vector molecule, a polycation compound such as polylysine, a receptor specific ligand, and, optionally, a psoralen inactivated virus such as Sendai or Adenovirus.

The first step of constructing one representative plasmid DNA Sindbis expression vector consists of digesting pKSS-INBV with Sac I, blunting with T4 polymerase, digesting with Sfi I, isolating the 2,689 bp fragment, and ligating into the pVGELVIS 10,053 bp vector fragment prepared by digestion with XbaI, blunting with T4 polymerase, digesting with Sfi I, treatment with CIAP, and 1% agarose/TBE gel electrophoresis. This construction is known as pVGELVIS-SINBV.

In order to insert the luciferase gene into the pVGELVIS-SINBV vector, the SV40 intron and transcription termination sequences at the 3'-end of luciferase must be removed so that when the pre-RNA, transcribed from the plasmid DNA luciferase vector after transfection into cells, is processed the 3'-end of the reporter gene is not separated from the Sindbis vector 3'-end. The Sindbis 5'- and 3'-ends contained within the pVGELVIS-SINBV vector are required in cis for the autocatalytic replication activity of the vector. The Sindbis vector 3'-end is required for initiation of synthesis of the antigenomic strand, which is the template for the subgenomic RNA encoding the heterologous or reporter protein.

The SV40 RNA processing signals positioned at the 3'-end of the luciferase gene are removed from the SIN-BV-luc construction described in section B above. The modified luciferase fragment is then placed in the pVGELVIS-SINBV construction described above via unique restriction sites. The alteration of the luciferase gene is accomplished with the primer pair shown below:

Forward primer 7328F (SIN nts 7328-7349):

---

5'-GTGGCGGATCCCTGAAAAGG  
(SEQ. ID NO. 10)

---

Reverse primer LucStop (buffer sequence/Not I, Xba I recognition sequences/pGL-2 nts 1725-1703):

---

5'-TATATGCGGCCGCTCTAGATTACAATTGGACITTCGCCC  
(SEQ. ID NO. 34)

---

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period. The amplification products are purified with GENECLAN™, digested with Xho I and Xba I, purified again with GENECLAN™, and the 2,037 bp fragment is ligated into the 13,799 bp fragment of pVGELVIS-SINBV resulting from digestion with Xho I and Xba I, and treatment with CIAP. This construction is known as pVGELVIS-SINBV-luc (abbreviated as ELVIS-luc).

The expression of luciferase in BHK-21 cells transfected with pVGELVIS-SINBV-luc DNA is measured in order to demonstrate that the Sindbis physical gene transfer vector is functional. Briefly, 5 ug of pVGELVIS-SINBV-luc DNA or 5 ug of in vitro transcribed RNA from linearized SINBV-luc template as described in section B, above, are complexed with 10 ul of LIPOFECTAMINE™ or LIPOFECTIN™, respectively, and transfected into 5x10<sup>5</sup> BHK-21 cells contained in 35 mM petri plates. The luciferase activity is determined from each of three samples at 2, 4, 8, 16, 20, 28, 48, 72, 96, and 120 hrs. post transfection. The results of this study, given in FIG. 9, demonstrate that the maximal level of reporter gene expression from the pVGELVIS-SINBV-luc vector is similar to that observed in cells transfected with in vitro transcribed RNA from linearized SINBV-luc template. However, the luciferase activity expressed from the pVGELVIS-SINBV-luc vector is at maximal levels at later time points compared to that observed with the SINBV-luc RNA vector, and continues at high levels while the activity from the RNA vector begins to diminish.

The following experiment is performed in order to demonstrate the level of enhancement of heterologous gene expression provided by the ELVIS vector system compared to the same RNA polymerase II promoter linked directly to the luciferase gene reporter. Briefly, the Sindbis NSPs are first deleted from the pVGELVIS-SINBV-luc vector in order to demonstrate the requirement for the viral enzymatic proteins for high levels of reporter gene expression. This is accomplished by digestion of pVGELVIS-SINBV-luc DNA with Bsp EI, purification with GENECLAN, and ligation under dilute conditions. This construction is deleted of nonstructural gene sequences between bases 422-7,054 and is analogous to the pVGSP6GENd1Bsp construction described in Example 3, section C above and shown schematically in FIG. 5. The construction described here is known as pVGELVIS-SINBVd1Bsp-luc (abbreviated as d1NSP ELVIS-luc). To link the luciferase gene directly to the MoMuLV LTR, the reporter is first inserted into the pCDNA3 vector (Invitrogen, San Diego, Calif.) between the Bam HI and Hind III sites. The luciferase fragment is derived from pGL2 plasmid exactly as described in Example 3 section B, above, and inserted into the 5428xbp fragment of pCDNA3 prepared by digestion with Hind III and Bam HI, treatment with CIAP, and purification on a 1% agarose/TBE gel. This construction is known as pCDNA3-luc. The U3 region of the MoMuLV LTR is amplified from the BAG vector using the PCR primers shown below as described in Example 2.

Forward primer: BAGBgl2F1 (buffer sequence/Bgl II recognition sequence/Mo-MLV LTR nts 1-22):

---

5'-TATATAGATCTAATGAAAGACCCACCTGTAGG  
(SEQ. ID NO. 15)

---

Reverse primer: BAGwt441R2 (SIN nts 5-1/Mo-MLV LTR nts 441-406):

---

5'-TCAATCCCCGAGTGAGGGGTTGTGGGCTCTTTTATTGAGC  
(SEQ. ID NO. 16)

---

The amplification products are purified with GENECLEAN and the ends are first blunted with T4 DNA polymerase, then digested with Bgl II, purified with GENECLEAN™ and ligated into the pCDNA3-luc plasmid prepared by digestion with Hind III, blunting with the Klenow enzyme and 50 uM dNTPs, digestion with Bgl II, and purification by 1% agarose/TBE gel electrophoresis. This construction is known as LTR-luc.

The plasmids ELVIS-luc, d1NSP ELVIS-luc, LTR-luc, and ELVIS-luc d1pro are each complexed with 10 ul of LIPOFECTAMINE™ and transfected into 5×10<sup>5</sup> BHK-21 cells contained in 35 mM petri plates. The luciferase activity is determined from each of three samples at 48 hrs. post-transfection. The results of this study, given in FIG. 10, demonstrate that the level of heterologous gene expression enhancement provided by the ELVIS system, compared to the same promoter linked directly to the heterologous gene is at least 10-fold. The comparatively low level of luciferase expression in cells transfected with the d1NSP ELVIS-luc construction demonstrates that the expression enhancement is a direct result of functional Sindbis NSPs. The autocata-

lytic amplification of the reporter gene mRNA as depicted in FIG. 8 provides a significant advantage in terms of levels of gene expression, compared to primary transcription from simple promoter-heterologous gene constructions. Thus, as shown schematically in FIG. 8, after transfection of the ELVIS vector primary transcription in the nucleus and transport of the vector RNA to the cytoplasm leads to the synthesis of Sindbis NSPs which catalyze the expansion of heterologous gene mRNA via an antigenome intermediate which in turn serves as the template for production of genomic and subgenomic mRNA.

An experiment is performed to demonstrate the expression and rescue of RNA- and plasmid DNA (ELVIS)-based Sindbis expression vectors. For the RNA vectors, 5×10<sup>5</sup> BHK-21 cells contained in 35 mM petri plates are transfected with SIN-luc RNA, or co-transfected with SIN-luc RNA and SINd1BspEI RNA, complexed with LIPOFECTAMINE™. For the ELVIS vectors, 5×10<sup>5</sup> BHK-21 cells contained in 35 mM petri plates are transfected with ELVIS-luc, or co-transfected with ELVIS-luc and pVGELVISd1BspEI, whose construction is described in Example 7, complexed with LIPOFECTAMINE™. The results of this study, shown

in FIG. 23 demonstrate clearly that the level of expression after transfection and transduction is similar between BHK cells co-transfected with RNA or ELVIS vectors. Thus, the ELVIS vectors are used not only as plasmid DNA expression vectors, but additionally expression and helper vector ELVIS constructs can be cotransfected into cells to generate recombinant vector particles.

#### F. CONSTRUCTION OF MODIFIED DNA-BASED ALPHAVIRUS EXPRESSION VECTORS

The overall efficiency of the ELVIS vector, as determined by level of heterologous gene expression, is enhanced by several modifications to the pVGELVIS-SINBV-luc vector. These modifications include alternate RNA polymerase II promoters and transcription termination signals, the addition of intron sequences and ribozyme processing signals in the vector construct, and substitution with a smaller plasmid vector backbone. The construction of these modified ELVIS vectors is detailed below.

The modified ELVIS vector is assembled on the plasmid vector pBGS131 (ATCC # 37443) which is a kanamycin resistant analogue of pUC 9 (Spratt et al., *Gene* 41:337-342, 1986). Propagation of pBGS 131 is in LB medium with 10 ug/ml kanamycin.

The transcription termination signals from the SV40 early region or Bovine growth hormone are inserted between the Sac I and Eco RI sites of pBGS131. The SV40 nts between viral nts 2643 to 2563 containing the early region transcription termination sequences are isolated by PCR amplification using the primer pair shown below and the pBR322/SV40 plasmid (ATCC # 45019) as template.

Forward primer SSVTT 2643 (buffer sequence/Sac I site/SV40 nts 2643-2613):

---

5'-TATATAGAGCTCTTACAAATAAAGCAATAGCATCACAATTTTC  
(SEQ. ID NO. 35)

---

Reverse primer RSVTT2563R (buffer sequence/Eco RI site/SV40 nts 2563-2588):

---

5'-TATATGAATTCGTTTGACAAAACCACTAGAAATG  
(SEQ. ID NO. 36)

---

The primers shown above are used in a PCR reaction with a three temperature cycling program as described throughout this example, using a 30 second extension period. The amplification products are purified with GENECLEAN™, digested with Sac I and Eco RI, purified again with GENECLEAN™, and the 90 bp fragment is ligated into the 3,655 bp fragment of pBGS 131 resulting from digestion with Sac I and Eco RI, and treatment with CIAP. This construction is known as pBGS131-3'SV40TT

The Bovine growth hormone transcription termination sequences are isolated by PCR amplification using the primer pair shown below and the pCDNA3 plasmid (Invitrogen) as template.

Forward primer BGHTTF (buffer sequence/Sac I site/pCDNA3 nts 1132-1161):

---

5'-TATATAGAGCTCTAATAAAATGAGGAAATTGCATCGCATTGTC  
(SEQ. ID NO. 37)

---

71

Reverse primer BGHTTR (buffer sequence/Eco RI site/  
pCDNA3 nts 1180-1154):

---

5'-TATATGAATTCATAGAATGACACCTACTCAGACAATGCGATGC  
(SEQ. ID NO. 38)

---

The primers shown above are used in a PCR reaction with a three temperature cycling program, using a 30 sec. extension period. The amplification products are purified with GENECLEAN™, digested with Sac I and Eco RI, purified again with GENECLEAN™, and the 58 bp fragment is ligated into the 3,655 bp fragment of pBGS131 resulting from digestion with Sac I and Eco RI, and treatment with CIAP. This construction is known as pBGS131-3'BGHTT.

In additional modifications to the ELVIS vector, the transcription termination sequences are fused directly to the 3'-end Sindbis sequences, resulting in deletion of the polyadenylate tract; or alternatively the antigenomic ribzyme sequence of hepatitis delta virus (HDV) is inserted between the 3'-polyadenylate tract of the ELVIS vector and the transcription termination signals.

The HDV ribozyme-containing construct is generated with PCR techniques and overlapping oligonucleotide primers which contain the minimal 84 nucleotide antigenomic ribozyme sequence (Perotta and Been, *Nature* 350:434-6, 1991). In addition to the HDV sequence, the primers contain flanking Sac I recognition sites for insertion at the 3' end of the ELVIS vector. The HDV ribozyme sequence is generated with the three overlapping primers shown below.

Forward primer SHDV1F (Buffer sequence/Sac I site/HDV RBZ seq.):

---

5'-TATATGAGCTCGGGTCGGCATGGCATCTCCAACCTCTCGCGGTCCG  
(SEQ. ID NO. 39)

---

Nested primer HDV17-68:

---

5'-TCCACCTCTCGGGTCGACCTGGGCATCGAAGGAGACGCACGTCCACT-3'  
(SEQ. ID NO. 40)

---

Reverse primer SHDV84R (Buffer sequence/Sac I site/HDV RBZ seq.):

---

5'-TATATAGAGCTCTCTCCTTAGCCATCGAGTGGACGTGCGTCTCTCTTC  
(SEQ. ID NO. 41)

---

The primers shown above are used in a PCR reaction with a three temperature cycling program as described throughout this example, using a 30 sec. extension period. The amplification products are purified with GENECLEAN™, digested with Sac I, purified again with GENECLEAN™, and the 94 bp fragment subsequently is ligated into plasmid vectors pBGS131-3'SV40TT or pBGS131-3'BGHTT that

72

are digested with SacI under limiting conditions that linearize (cut 1 of 2 sites) and are treated with CIAP. These constructions are known as pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT. Insertion of the HDV ribozyme in both the correct orientation and in the correct Sac I site is determined by sequencing. In addition, longer or shorter HDV ribozyme sequences, or any other catalytic ribozyme sequence, may be readily substituted given the disclosure provided herein.

In the second vector 3'-end configuration, the SV40 or BGH transcription termination signals are fused directly to the 3'-end of the ELVIS vector corresponding to Sindbis nt 11,700 and the polyadenylate tract is deleted. This construction is accomplished according to the steps outlined above in Example 3, sections A and B for the assembly of the pKSSINBV and pKSSINBV-luc vectors. However, in this application the vector 3'-end primer does not contain a 25 polyadenylate tract. The 3'-end of the vector is synthesized with the primer pair shown below:

Forward Primer: SIN11664F: (buffer sequence/Not I site/  
SIN nts 11664-11698):

---

5'-TATATGCGGCCGCTTCTTTTATTAATCAACAAAATTTGTGTTTAA  
(SEQ. ID NO. 42)

---

Reverse Primer: SSIN11700R (buffer sequence/Sac I site/  
SIN nts 11700-11655):

---

5'-TATATGAGCTCGAAATGTTAAAAACAAAATTTGTGTG  
(SEQ. ID NO. 43)

---

The primers shown above are used in a PCR reaction with a three temperature cycling program as described throughout this example, using a 30 sec. extension period. Assembly of the pKSSINBV and pKSSINBV-luc vectors is precisely as

shown in Example 3, sections A and B. These constructions are known as pKSSINBVd1A and pKSSINBVd1A-luc.

60

The ELVIS expression vectors are assembled further onto the various 3' end processing plasmid constructions described above. The Sindbis vectors containing a polyadenylate tract are combined with the plasmid constructions containing the HDV ribozyme sequence and the SV40 or BGH transcription termination signals. This construction corresponds to the insertion of pKSSINBV and pKSSINBV-

luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids. Alternatively, the Sindbis vectors terminating precisely at the viral 3' end corresponding to viral nt 11,700 are linked directly to the SV40 or BGH transcription termination signals. This construction corresponds to the insertion of pKSSINBVd1A and pKSSINBVd1A-luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids.

The Sindbis vectors pKSSINBV and pKSSINBV-luc are digested with Sac I and Bgl II, and the 5,522 bp (pKSSINBV) or 8211 bp (pKSSINBV-luc) fragments are purified by 1% agarose/TBE gel electrophoresis and inserted into the linearized pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids prepared by digestion with Sac I and Bgl II and treatment with CIAP. These constructions are known as:

pBGS131/d1proSINBV-luc/HDV/3'SV40TT  
pBGS131/d1proSINBV-luc/HDV/3'BGHTT  
pBGS131/d1proSINBV/HDV/3'SV40TT  
pBGS131/d1proSINBV/HDV/3'BGHTT

Using the same strategy described above, the Sindbis vectors pKSSINBVd1A and pKSSINBVd1A-luc are digested with Sac I and Bgl II, and the 5,497 bp (pKSSINBVd1A) or 8186 bp (pKSSINBVd1A-luc) fragments are purified by 1% agarose/TBE gel electrophoresis and inserted into the linearized pBGS131/3'SV40TT and pBGS 131/3'BGHTT plasmids prepared by digestion with Sac I and Bgl II and treatment with CIAP. These constructions are known as:

pBGS131/d1proSINBV-luc/3'SV40TT  
pBGS131/d1proSINBV-luc/3'BGHTT  
pBGS131/d1proSINBV/3'SV40TT  
pBGS131/d1proSINBV/3'BGHTT

The addition of an RNA polymerase II promoter and Sindbis nucleotides 1–2289 is the last step required to complete the construction of the modified ELVIS expression vectors of the eight constructions shown below:

pBGS131/d1proSINBV-luc/HDV/3'SV40TT  
pBGS131/d1proSINBV-luc/HDV/3'BGHTT  
pBGS131/d1proSINBV/HDV/3'SV40TT  
pBGS131/d1proSINBV/HDV/3'BGHTT  
pBGS131/d1proSINBV-luc/3'SV40TT  
pBGS131/d1proSINBV-luc/3'BGHTT  
pBGS131/d1proSINBV/3'SV40TT  
pBGS131/d1proSINBV/3'BGHTT

These eight constructions contain a unique Bgl II restriction site, corresponding to Sindbis nt 2289. The RNA polymerase II promoter and Sindbis nucleotides 1–2289 are inserted into these constructions by the overlapping PCR technique described for the pVGELVIS construction in Example 2. In order to insert the RNA polymerase II promoter and the 2289 Sindbis nts, the eight constructions shown above are digested with Bgl II and treated with CIAP.

The U3 region of the long terminal repeat (LTR) from Moloney murine leukemia virus (Mo-MLV) is positioned at the 5' viral end such that the first transcribed nucleotide is a single G residue, which is capped in vivo, followed by the Sindbis 5' end. Amplification of the Mo-MLV LTR in the first primary PCR reaction is accomplished in a reaction containing the BAG vector (Price et al., *PNAS* 84:156–160, 1987) and the following primer pair:

Forward primer: BAGBgl2F1 (buffer sequence/Bgl II recognition sequence/Mo-MLV LTR nts 1–22):

5'-TATATAGATCTAATGAAAGACCCACCTGTAGG  
(SEQ. ID NO. 15)

Reverse primer: BAGwt441R2 (SIN nts 5–1/Mo-MLV LTR nts 441–406):

5'-TCAATCCCGAGTGAGGGGTGTGGGCTCTTTATTGAGC  
(SEQ. ID NO. 16)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 30 second extension period.

Amplification of the Sindbis 5' end in the second primary PCR reaction is accomplished in a reaction containing the pVGSP6GENrep clone and the following primer pair: Forward primer: (Mo-MLV LTR nts 421–441/SIN nts 1–16):

5'-CCACAACCCCTCACTCGGGGATTGACGGCGTAGTAC  
(SEQ. ID NO. 17)

Reverse primer: (SIN nts 3182–3160):

5'-CTGGCAACCGGTAAGTACGATAC  
(SEQ. ID NO. 18)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period.

The 457 bp and 3202 bp products from the primary PCR reactions are purified with GENECLAN™, and used together in a PCR reaction with the following primer pair: Forward primer: BAGBgl2F1 (buffer sequence/Bgl II recognition sequence/Mo-MLV LTR nts 1–22):

5'-TATATAGATCTAATGAAAGACCCACCTGTAGG  
(SEQ. ID NO. 15)

Reverse primer: (SIN nts 2300–2278):

5'-GGTAACAAGATCTCGTGCCGCTG  
(SEQ. ID NO. 19)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period. The 25 3'-terminal bases of the first primary PCR amplicon product overlap with the 25 5'-terminal bases of the second primary PCR amplicon product; the resultant 2,752 bp overlapping secondary PCR amplicon product is purified by 1% agarose/TBE electrophoresis, digested with Bgl II, and the 2,734 bp product is ligated into the eight ELVIS constructions described above. These constructions are named as shown below:

MpLTRELVIS-luc/D/S  
MpLTRELVIS-luc/D/B  
MpLTRELVIS/D/S  
MpLTRELVIS/D/B  
MpLTRELVIS-luc/S  
MpLTRELVIS-luc/B  
MpLTRELVIS/S

**MpLTRELVIS/B**

Using the same overlapping PCR approach, the CMV promoter is positioned at the 5' viral end such that transcription initiation results in the addition of a single non-viral nucleotide at the Sindbis 5' end. Amplification of the CMV promoter in the first primary PCR reaction is accomplished in a reaction containing the pCDNA3 plasmid and the following primer pair:

Forward primer: pCBgl233F (buffer sequence/Bgl II recognition sequence/CMV promoter nts 1–22):

---

5'-TATATATAGATCTTTGACATTGATTATTGACTAG  
(SEQ. ID NO. 44)

---

Reverse primer: SNCMV1142R (SIN nts 8–1/CMV pro nts 1142–1108):

---

5'-CCGTCAATACGGTTCACTAAACGAGCTCTGCTTATATAGACC  
(SEQ. ID NO. 45)

---

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 1 minute extension period.

Amplification of the Sindbis 5' end in the second primary PCR reaction is accomplished in a reaction containing the pVGSP6GENrep clone and the following primer pair:

Forward primer: CMVSIN1F (CMV pro nts 1124–1142/SIN nts 1–20):

---

5'-GCTCGTTTAGTGAACCGTATTGACGGCGTAGTACACAC  
(SEQ. ID NO. 46)

---

Reverse primer: (SIN nts 3182–3160):

---

5'-CTGGCAACCGGTAAAGTACGATAC  
(SEQ. ID NO. 18)

---

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period.

The 600 bp and 3200 bp products from the primary PCR reactions are purified with GENECLAN™, and used together in a PCR reaction with the following primer pair: Forward primer: pCBgl233F (buffer sequence/Bgl II recognition sequence/CMV promoter nts 1–22):

---

5'-TATATATAGATCTTTGACATTGATTATTGACTAG  
(SEQ. ID NO. 44)

---

Reverse primer: (SIN nts 2300–2278):

---

5'-GGTAACAAGATCTCGTGCCGTG  
(SEQ. ID NO. 19)

---

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period.

The 26 3' terminal bases of the first primary PCR amplification product overlaps with the 26 5' terminal bases of the second primary PCR amplicon product; the resultant 2,875 bp overlapping secondary PCR amplicon product is purified by 1% agarose/TBE electrophoresis, digested with Bgl II,

and ligated into the four ELVIS constructions described above. These constructions are named as shown below:

MpCMVELVIS-luc/D/S

MpCMVELVIS-luc/D/B

MpCMVELVIS/D/S

MpCMVELVIS/D/B

MpCMVELVIS-luc/S

MpCMVELVIS-luc/B

MpCMVELVIS/S

MpCMVELVIS/B

Using the same overlapping PCR approach, the SV40 early region promoter is positioned at the 5' viral end such that the major cap site of transcription initiation results in the addition of a single non-viral nucleotide at the Sindbis 5' end. Amplification of the SV40 promoter in the first primary PCR reaction is accomplished in a reaction containing the pBR322/SV40 plasmid (ATCC # 45019) and the following primer pair:

Forward primer: B2SVpr250F (buffer sequence/Bgl II recognition sequence/SV40 nts 250–231):

---

5'-TATATATAGATCTGGTGTGGAAAGTCCCCAGGC  
(SEQ. ID NO. 47)

---

Reverse primer: SINSV5235R (SIN nts 13–1/SV40 nts 5235–10):

---

5'-CTACGCCGTCAATGCCGAGGCGGCTCGGCC  
(SEQ. ID NO. 48)

---

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 30 second extension period.

Amplification of the Sindbis 5' end in the second primary PCR reaction is accomplished in a reaction containing the pVGSP6GENrep clone and the following primer pair:

Forward primer: SVSIN1F (SV40 nts 3–5235/SIN nts 1–25):

---

5'-GGCCGCCCTCGGCATTGACGGCGTAGTACACACTATTG  
(SEQ. ID NO. 49)

---

Reverse primer: (SIN nts 3182–3160):

---

5'-CTGGCAACCGGTAAAGTACGATAC  
(SEQ. ID NO. 18)

---

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period.

The 280 bp and 3,194 bp products from the primary PCR reactions are purified with GENECLAN™, and used together in a PCR reaction with the following primer pair:

Forward primer: B2SVpr250F (buffer sequence/Bgl II recognition sequence/SV40 nts 250–231):

---

5'-TATATATAGATCTGGTGTGGAAAGTCCCCAGGC  
(SEQ. ID NO. 47)

---



Reverse primer: (SIN nts 2300-2278):

---

5'-GGTAACAAGATCTCGTGCCGTG  
(SEQ. ID NO. 19)

---

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period.

The 25 3' terminal bases of the first primary PCR amplicon product overlaps with the 25 5' terminal bases of the second primary PCR amplicon product; the resultant 2,543 bp overlapping secondary PCR amplicon product is purified by 1% agarose/TBE electrophoresis, digested with Bgl II, and ligated into the four ELVIS constructions described above. These constructions are named as shown below:

MpSV40ELVIS-luc/D/S  
MpSV40ELVIS-luc/D/B  
MpSV40ELVIS/D/S  
MpSV40ELVIS/D/B  
MpSV40ELVIS-luc/S  
MpSV40ELVIS-luc/B  
MpSV40ELVIS/S  
MpSV40ELVIS/B

The luciferase expression levels, after transfection of BHK-21 cells, are determined with each of the reporter gene containing complete modified ELVIS constructions detailed above, in order to determine the optimal desired configuration. The heterologous gene is inserted into the multiple cloning site of the ELVIS vector, as described for the insertion of the luciferase gene in Example 3, section B.

In order to increase the efficiency of the ELVIS system, in terms of functional vector RNA transported to the cytoplasm per nuclear DNA template, the SV40 small t antigen intron can be inserted into the ELVIS expression vectors. Insertion of the SV40 small t antigen intron sequences into the Xho I site immediately downstream of the 5' Sindbis sequences is accomplished by limited digestion (cut 1 of 2 sites); or, alternatively at the unique Not I site immediately upstream of the 3' Sindbis sequences.

For insertion into the Xho I site of the ELVIS vectors, amplification of the SV40 small t antigen intron sequences is accomplished in a reaction containing the pBR322/SV40 plasmid (ATCC # 45019) and the following primer pair:  
Forward primer: XSVSD4647F (buffer sequence/Xho I recognition sequence/SV40 nts 4647-4675):

---

5'-TATATATCTCGAGAAGCTCTAAGGTAAATATAAAATTACC  
(SEQ. ID NO. 50)

---

Reverse primer: XSVSA4562R (buffer sequence/Xho I recognition sequence/SV40 nts 4562-4537):

---

5'-TATATATCTCGAGAGGTGGAATCTAAATACACAAAC  
(SEQ. ID NO. 51)

---

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 30 second extension period. The amplification products are purified with GENECLAN™, digested with Xho I, re-purified with GENECLAN™ and inserted into Xho I linearized (by limited digest) and CIAP treated complete modified ELVIS vectors described above. Insertion of the SV40 small t antigen intron in the correct orientation in the ELVIS vector is determined by sequencing.

For insertion into the Not I site of the ELVIS vectors, amplification of the SV40 small t antigen intron sequences is accomplished in a reaction containing the pBR322/SV40 plasmid and the following primer pair:

Forward primer: NSVSD4647F (buffer sequence/Not I recognition sequence/SV40 nts 4647-4675):

---

5'-TATATATGCGGCGCAAGCTCTAAGGTAAATATAAAATTACC  
(SEQ. ID NO. 52)

---

Reverse primer: XSVSA4562R (buffer sequence/Not I recognition sequence/SV40 nts 4562-4537):

---

5'-TATATATGCGGCGCAGGTGGAATCTAAATACACAAAC  
(SEQ. ID NO. 53)

---

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 30 second extension period. The amplification products are purified with GENECLAN™, digested with Not I, re-purified with GENECLAN™ and inserted into Not I linearized and CIAP treated complete modified ELVIS vectors described above. Insertion of the SV40 small t antigen intron in the correct orientation in the ELVIS vector is determined by sequencing. Alternatively, the SV40 small t antigen may be inserted at other sites within the ELVIS vector, which do not impair function of the vector, using the disclosure provided herein.

The luciferase expression levels, after transfection of BHK-21 cells with the SV40 small t antigen intron containing ELVIS vectors, are assayed in order to determine the optimal desired configuration. The heterologous gene is inserted into the multiple cloning site of the ELVIS vector, as described for the insertion of the luciferase gene in Example 3, section B.

A linker sequence is inserted into the pKSSINBV and into the pVGELVIS-SINBV constructs to facilitate the insertion of heterologous sequences. The linker is constructed using two complementary 35nt oligonucleotides that form a duplex with Xho I and Xba I compatible sticky ends when hybridized.

---

SINBVLinkF: 5'TCGAGCAOGTGGCGCGCCTGATCACGCGTAGGCCT (SEQ. ID NO. 54)  
SINBVLinkR: 5'CTAGAGGCTACGCGTGATCAGGCGGCCACGTGC (SEQ. ID NO. 55)

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60

The oligonucleotides are phosphorylated with T4 polynucleotide kinase, heated to 90° C., and slow cooled to allow hybridization to occur. The hybrid is then ligated to the 10.6 kb fragment of pKSSINBV-Luc obtained after digestion with XhoI and XbaI, followed by treatment with alkaline phosphatase and agarose gel purification. The resulting construct contains Xho I, Pml I, Asc I, Bcl I, Mlu I, Stu I,

65

Xba I, and Not I as unique sites between the Sindbis junction region and the Sindbis 3' end. This construct is known as pKSSINBV-Linker.

This linker also is cloned into the pVGELVIS-SINBV constructs. The linker is inserted by digestion of pVGELVIS-SINBV-luc with Sfi I and Not I. The 10.1 kb fragment is agarose gel purified, and this fragment was ligated to the gel purified 2.6 kb fragment from a Sfi I/Not I digest of pKSSINBV-Linker. The resulting construct contains Xho I, Pml I, Asc I, Mlu I, and Not I as unique sites between the Sindbis junction region and the Sindbis 3' end. This construct is known as pVGELVIS-SINBV-Linker.

Additional experiments are performed to compare the relative expression activities of Sindbis RNA and DNA reporter vectors in transfected BHK cells (FIG. 22). Luciferase expression is approximately 30-fold higher in cells transfected with in vitro transcribed SIN-luc RNA, compared to the level in cells transfected with ELVIS-luc plasmid DNA (FIG. 22A). The data also demonstrate that direct linkage between the Sindbis virus 3'-end and two different transcription termination/polyadenylation signals, resulting in deletion of the synthetic A<sub>25</sub> tract, decreased the activity of the DNA vector by more than three orders of magnitude (FIG. 22A). However, measurable expression of luciferase is detected, suggesting that these 3' end modified Sindbis DNA vectors do function in transfected cells at some low level. Additionally, the insertion of a HDV ribozyme processing sequence, downstream of the A<sub>25</sub> tract, increases activity of the DNA vector 3-4 fold over the ELVIS-luc vector or an analogous construct with the HDV ribozyme inserted in a reverse orientation (FIG. 22B).

Based on the decreased expression levels observed when the synthetic A<sub>25</sub> tract is deleted, additional constructs related to MpELVIS/S and MpELVIS/B are then made exactly as outlined in the above example utilizing the Sindbis sequences from the pKSSINBV and pKSSINBV-luc vectors to include the A<sub>25</sub> tract. These constructions are named as shown below:

MpLTRELVIS-luc/A/S  
MpLTRELVIS-luc/A/B  
MpLTRELVIS/A/S  
MpLTRELVIS/A/B  
MpCMVELVIS-luc/A/S  
MpCMVELVIS-luc/A/B  
MpCMVELVIS/A/S  
MpCMVELVIS/A/B  
MpSV40ELVIS-luc/A/S  
MpSV40ELVIS-luc/A/B  
MpSV40ELVIS/A/S  
MpSV40ELVIS/A/B

#### G. REPORTER GENE EXPRESSION IN RODENTS INOCULATED INTRAMUSCULARLY WITH ELVIS VECTORS

Using techniques described above, the lacZ gene encoding the  $\beta$ -galactosidase reporter protein was cut from the plasmid pSV- $\beta$ -galactosidase (PROMEGA CORP, Madison, Wis.) and substituted into the ELVIS-luc plasmid DNA vector in place of luciferase. To examine in vivo gene expression from ELVIS vectors, Balb/c mice and rats are injected intramuscularly (i.m.) with ELVIS- $\beta$ -gal or ELVIS-luc plasmid DNA vectors. FIG. 24 demonstrates the in vivo expression of  $\beta$ -galactosidase in muscle tissue taken from a rat and stained with X-gal at three days post i.m. injection. Mice injected with ELVIS- $\beta$ -gal also demonstrate positively staining blue muscle fibers. Luciferase expression levels from muscle which were between 75- and 300-fold higher than control levels were detected in 3/4 Balb/c mice at two

days post i.m. inoculation with ELVIS-luc plasmid. In other experiments, C3H/HeN mice were injected i.m. with ELVIS vectors expressing either the hepatitis B virus core (HBV-core) or hepatitis B virus e (HBV-e) proteins. Using ELISA detection systems, both HBV-core- and HBV-e-specific IgG antibodies were detected in serum samples collected from the mice 10 days following the second injection with the vectors. These experiments demonstrate that Sindbis-derived DNA vectors are able to express foreign genes in vivo, in rat and mouse muscle.

#### H. ADAPTATION OF ALPHAVIRUS EXPRESSION VECTORS

The following description details how to identify alphaviral vectors according to the invention adapted to grow in cells of a particular eukaryotic species. Specifically, adaptation of Sindbis virus variants adapted to grow in human cells is disclosed. As those in the art will appreciate, the following procedure can be employed to adapt other alphaviral vectors to particular eukaryotic species.

To adapt Sindbis viral vectors derived from BHK-21 cells to human cells, Sindbis viral vectors produced in accordance with this invention are propagated by serial passage in HT1080 (ATCC acc. no. CCL 121) and DM150 (a human cell line established from a primary melanoma tumor) cell lines in order to select variants which are able to establish high titer productive infections in human cells. Isolation of Sindbis variants adapted to human cells is accomplished by the following method: HT1080 and DM150 cells propagated in DMEM with 10% fetal calf serum (FCS) are infected at a multiplicity of infection of 5 with the virus contained in a small volume to facilitate infection. At one hour post infection, the inoculum is removed, the monolayer washed several times with DMEM, and the media replenished. The viral supernatant is harvested at 7 hours post infection, clarified by centrifugation, and divided into three aliquots. Two aliquots are frozen and the other aliquot is split and used to infect fresh HT1080 and DM150 monolayers. This process is repeated at least 10 times or as sufficient to generate variants which replicate efficiently in human cells. After each serial passage, plaque assays are performed in BHK cells or the homologous cell line in which the virus was propagated to determine an increase in virus titer in human cell lines. Sindbis variants adapted to human cells which contain the highest level of virus produced during serial HT1080 or DM150 cell line passage are then isolated from supernatants by three rounds of plaque purification. The phenotype of the plaque purified human variant is verified by determining its growth properties in human cell lines.

In an alternative approach, variants which are able to establish high titer productive infections in human cells are isolated by plaque morphology. Human cell lines, for example HT1080 and DM150, are infected at low multiplicity of infection with Sindbis virus grown in BHK-21 cells and overlaid with agar. At 24-30 hours post infection, large plaques, indicative of variants able to propagate efficiently in human cells, are picked. The variants are then purified by two additional serial rounds of plaque purification. The phenotype of candidate Sindbis variants can then be determined by comparing growth properties on human and BHK-21 cells with BHK-21 cell-propagated Sindbis virus.

Another similar approach enables the production of Sindbis variants which establish high titer persistent, i.e., noncytotoxic, infection of human cells. Specifically, human cells are infected with a Sindbis virus preparation containing a high percentage of defective interfering (DI) particles

isolated by undiluted serial passage in HT1080 or DM150 cells. Cells which survive infection with this D1 contaminated Sindbis stock are allowed to proliferate. Virus is isolated from the supernatant and purified by multiple rounds of plaque purification in BHK-21 or human cells. The desired phenotype of the Sindbis variant is verified by determining its ability to establish persistent noncytotoxic persistent infection in human cell lines.

Following identification of one or more Sindbis variants having the desired phenotype, purified viral RNA from the Sindbis variant is cloned and characterized in order to identify the nonstructural and structural genes and noncoding region changes which contribute to the observed phenotype. Sindbis variant genomic cDNA cloning is accomplished by RT-PCR, as detailed in Example 1 and the phenotype of the molecularly cloned virus strains is verified.

Viral genetic determinants can be mapped by identifying at what level Sindbis infection of human cells is inhibited, i.e., at the stage of adsorption, entry, replication, or assembly. The 5'-end, junction region, and nonstructural and noncoding region genetic determinants responsible for human variant phenotypes can be mapped by exchanging defined regions from pKSSINBV-luc, supra, with corresponding regions from the variant cDNA to produce various "test" SIN-luc vectors. After packaging by co-transfection, the level of luciferase expression in DM150, HT1080, and BHK cells infected with either pKSSINBV-luc or the "test" SIN-luc vector is compared. Exchanging defined regions between vectors may be accomplished by exploiting convenient restriction endonuclease recognition sites, for example (Viagene SIN-BV numbering): Afl II (4573), Age I (3172, 6922), Avr II (4281), Bgl II (2289), BpuII02I (5602, 6266), BsaBI (2479) BsiBI (4706, 6450), Eco47III (1407), Hpa I (6920), Mun I (42, 2785), Nru I (2324), Nsi I (2006, 6462), PflMI (4374), Sfi I (5122), and XhoI (7645). Precise nucleotide identification of genetic determinants resulting in the human variant phenotype can be accomplished by sequencing.

The 3'-end nonstructural and coding region genetic determinants responsible for the variant phenotype may be mapped by exchanging defined regions with the d1-BspEI cotransfection packaging vector. After packaging by co-transfection, the level of luciferase expression in DM150, HT1080, and BHK cells infected with pKSSINBV-luc packaged with the d1-BspEI cotransfection packaging vector or with the "test" d1-BspEI cotransfection packaging vector is compared. Exchanging defined regions between vectors may be accomplished by exploiting convenient restriction endonuclease recognition sites, for example (Viagene genomic Sindbis numbering): AatII (8000), Afl II (7969, 8836), Aval (9414), BclI (9356), BpuII02I (8911), BsiWI (10379), BspMII (7054), Bsu36I (8892), EcoNI (10048, 10923), EcoRI (9077), KasI (10036, 11308), NruI (8329), PflMI (9554), PmlI (8070), SalI (9589, 11085), SmaI (9416), SplI (10379), StuI (8572), and (9414). Precise nucleotide identification of genetic determinants resulting in the human variant phenotype can be accomplished by sequencing.

#### I. RECOMBINANT PROTEIN EXPRESSION

The eukaryotic layered vector initiation systems of the invention can be used to direct the expression of one or more recombinant proteins in transformed or transfected eukaryotic host cells. A representative example of a recombinant protein which may be expressed using a eukaryotic layered vector initiation system is insulin.

The gene encoding human insulin was identified in 1980 by Bell, et al. [*Nature*, vol. 284, pp. 26-32]. The entire coding region for human preproinsulin (hppi) can be cloned

from a variety of sources, e.g., a human pancreatic cDNA library [Clontech, Palo Alto, Calif., catalog no. HL1163a] using standard PCR techniques. Primers for amplifying the coding region flank the 5' and 3' ends of the gene. The 5' primer includes an XhoI site and the 3' primer includes a NotI recognition sequence. After PCR amplification, the reaction products are purified using GENE CLEAN™, followed by XhoI and NotI digestion. The DNA is then gel purified and ligated into XhoI/NotI cleaved, CIAP-treated pVGVIS-SINBV, infra, to make pELVS-hppi.

Alternatively, the hppi amplicon is inserted into Xho I/Not I cleaved, CIAP-treated SIN-BV, infra, to make pSIN-BV-hppi. RNA from Sac I-linearized pSIN-BV-hppi plasmid is synthesized in vitro as described in Example 3. Production of SIN-BV-hppi recombinant vector particles is accomplished by transfection of LIPOFECTIN™-complex SIN-BV-hppi RNA into the Sindbis vector packaging cell lines as described in Example 7. Generation of vector particles having expression vectors derived from Sindbis variants which establish high titer persistent noncytotoxic infection of human cells is accomplished by the same procedure.

pELVS-hppi is then introduced (e.g., by electroporation or by complexing with lipofectamine) into a suitable eukaryotic host cell, preferably an undifferentiated cell, for instance, F9 cells, infra. The transformed cells are then grown in the presence of G418 under suitable nutrient conditions (i.e., an appropriate medium, such as DMEM, including any required supplements, at 37° C.). The cells can be grown in a variety of formats, including in roller bottles, cell hotels, and bioreactors. Recombinant protein production is initiated by adding retinoic acid or another suitable inducing agent to the medium. At 12 to 48 hours post-vector induction, the optimal level of insulin is expressed into the medium and is recovered according to techniques known in the art. The insulin is recovered from the cell supernatants up to 18 hrs from the time in which the vector establishes a cytotoxic infection. Recovery of insulin from cells infected with expression vectors derived from Sindbis human cell variants may be harvested over a period extending to 3-5 days post induction. Insulin so produced is recovered according to techniques known in the art. The isolated recombinant protein may then be formulated in any of a number of pharmaceutical compositions suitable for human administration.

#### J. LYOPHILIZED EUKARYOTIC LAYERED VECTOR INITIATION SYSTEM VACCINES

One aspect of the invention concerns the use of eukaryotic layered vector initiation systems according to the invention as vaccines to immunize a human patient's or non-human animal's immune system against a particular disease. Such vaccines can be employed either prophylactically or therapeutically to prevent or treat disease. Diseases which may be treated with such vaccines include those caused by various pathogenic agents, such as procaryotic or eukaryotic microorganisms or viruses, or cancer.

For example, each of the vector constructs described herein and containing the heterologous sequence of a suitable antigen is readily lyophilized for long term stability. Upon re-hydration in an appropriate diluent, administration is performed and subsequent expression occurs. Additional alphavirus vector constructs not disclosed in the present invention, including those described in the literature (see Hahn et al., *Proc Natl Acad Sci USA* 89: 2679-2683, 1992) are readily convertible to a eukaryotic layered vector initiation system format by those skilled in the art and using the knowledge provided herein. Conversion of transient alphavirus vector systems to the format of a eukaryotic

layered vector initiation system thus modify the duration of heterologous sequence expression to that of a more permanent and stable expression system. Advantages of this permanent and stable system include longer term expression, allowing greater prophylactic and therapeutic effects in both medical and veterinary applications.

#### K. EUKARYOTIC LAYERED VECTOR INITIATION SYSTEMS FOR PLANT APPLICATIONS

Given the disclosures provided herein, the adaptation of eukaryotic vector initiation system technologies to plant application is readily performed by those skilled in the art. For illustration purposes, any of several positive-stranded plant viruses (for example, potato virus X (PVX, Huisman et al., *J. Gen. Virol.* 69:1789-1798, 1988), tobacco mosaic virus (TMV, Goelet et al., *Proc. Natl. Acad. Sci. USA* 79:5818-5822, 1982), and tobacco etch virus (TEV, Allison et al., *Virology* 154:9-20, 1986), see also, specifications) may be converted to a cDNA form using PCR and specific oligonucleotide primers, chosen from published sequences, as described in Example 1. After assembly of a full-length genomic clone linked to a bacteriophage RNA polymerase promoter, and determination of infectivity of in vitro synthesized transcripts, the cDNA is exchanged into a vector containing an RNA polymerase II promoter and transcription termination/polyadenylation sequence, as described in Example 2. For plant applications, such promoter and termination sequences are chosen from the appropriate plant systems (e.g., CaMV 35S promoter (Guilley et al., *Cell* 30:763-773, 1982), and nopaline synthase promoter and transcription termination sequence (Sanders et al., *Nucleic Acids Res.* 15:1543-1558). Vector constructs derived from these infectious genomic cDNA clones is subsequently accomplished using any of the approaches described in the present invention (e.g., use of subgenomic promoters, replacement of structural protein genes, use of IRES sequences). Specific applications of such plant eukaryotic layered vector initiation systems may include, but are not limited to, the expression of host-derived resistance sequences, pathogen-derived resistance sequences (e.g., protein-encoding, nonprotein-encoding, and defective interfering sequences), and growth promoting sequences, by the creation of transgenic plants harboring such systems.

#### L. TRANSGENIC ANIMAL APPLICATIONS

In accordance with the non-parenteral administration the present invention, the gene delivery vehicles, particularly those comprised of unencapsidated nucleic acid, may be complexed with a polycationic molecule to provide polycation-assisted non-parenteral administration. Such a method of gene delivery facilitates delivery of a gene via mediation by a physical particle comprised of multiple components that augment the efficiency and specificity of the gene transfer. In particular, polycationic molecules, such as polylysine and histone, have been shown to neutralize the negative charges on a nucleic acid molecule and to condense the molecule into a compact form. This form of molecule is transferred with high efficiency in cells, apparently through the endocytic pathway. The uptake in expression of the nucleic acid molecule in the host cell results after a series of steps, as follows: (1) attachment to cell surface; (2) cell entry via endocytosis or other mechanisms; (3) cytoplasmic compartment entry following endosome release; (4) nuclear transport; and (5) expression of the nucleic acid molecule carried by the gene delivery vehicle. In a further preferred embodiment, multi-layer technologies are applied to the polycation-nucleic acid molecule complex to facilitate completion of one or more of these steps. For example, a ligand such as asialoglycoprotein, transferrin, and immuno-

globulin may be added to the complex to facilitate binding of the cell complex to the cell surface, an endosomal disruption component (e.g., a viral protein, a fusogenic peptide such as the n-terminus of the influenza virus hemagglutinin or an inactivated virus) is added to facilitate the release of DNA from the endosome, or a nuclear protein (or a peptide containing a nuclear localization signal) is added to facilitate the transport of the DNA into the nucleus. In a further preferred embodiment, the composition comprising the complex includes inactivated adenovirus particles (Curiel, D. T., et al., *PNAS* 88: 8850-8854, 1991; Cristiano, R. J., *PNAS* 90: 2122-2126 1993; Cotten, M., et al., *PNAS* 89: 6094-6098 1992; Lozier, J. N., et al., *Human Gene Therapy* 5: 313-322, 1994; Curiel, D. T., et al., *Human Gene Therapy* 3: 147-154, 1992; Plank, C. et al., *Bioconjugate Chem.* 3: 533-539, 1992; Wagner, E. et al., *PNAS* 88: 4255-4259, 1991). The assorted components comprising the multi-layer complex may be varied as desired, so that the specificity of the complex for a given tissue, or the gene expressed from the gene delivery vehicle, may be varied to better suit a particular disease or condition.

As noted above, various methods may be utilized to administer gene delivery vehicles of the present invention, including nucleic acids which encode the immunogenic portion(s) discussed above, to warm-blooded animals such as humans, directly. Suitable methods include, for example, various physical methods such as direct DNA injection (Acsadi et al., *Nature* 352:815-818, 1991), and microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991).

Within an in vivo context, the gene delivery vehicle can be injected into the interstitial space of tissues including muscle, brain, liver, skin, spleen or blood (see, WO 90/11092). Administration may also be accomplished by intravenous injection or direct catheter infusion into the cavities of the body (see, WO 93/00051), discussed in more detail below.

It is generally preferred that administration of the gene delivery vehicles at multiple sites be via at least two injections. In this regard, suitable modes of administration include intramuscular, intradermal and subcutaneous injections, with at least one of the injections preferably being intramuscular. In particularly preferred embodiments, two or more of the injections are intramuscular. However, although administration via injections is preferred, it will be evident that the gene delivery vehicles may be administered through multiple topical or separate ocular administrations. Further, a number of additional routes are suitable for use within the present invention when combined with one or more of the routes briefly noted above, including intraperitoneal, intracranial, oral, rectal, nasal, vaginal and sublingual administration. Methods of formulating and administering the gene delivery vehicles at multiple sites through such routes would be evident to those skilled in the art and are described in U.S. Ser. No. 08/366,788 and U.S. Ser. No. 08/367,071 incorporated herein by reference in their entirety.

#### M. VETERINARY APPLICATIONS

From the description provided herein, those skilled in the art will appreciate that the alphavirus vector constructs, recombinant alphavirus particles, and eukaryotic layered vector initiation systems provided by the present invention can also be readily utilized in non-human animal (e.g., veterinary) applications. Such applications may include prophylactics (e.g., vaccines), immunotherapeutics, and palliatives. Within such aspects, compositions and methods are provided for administering an alphavirus vector construct,

recombinant alphavirus particle, or eukaryotic layered vector initiation system which is capable of preventing, inhibiting, stabilizing or reversing infectious diseases in non-human animals.

Specifically, within one aspect of the present invention, compositions and methods are provided for stimulating an immune response (either humoral or cell-mediated) to a pathogenic agent, such that the pathogenic agent is either killed or inhibited. Representative examples of pathogenic agents of veterinary importance include bacteria, fungi, parasites and viruses.

More specifically, sequences which encode immunoreactive polypeptides of the pathogenic agents may, in certain embodiments, be chosen from a group that includes the Bunyaviridae (e.g., Rift Valley Fever virus (Giorgi et al., *Virology* 180:738-753, 1991; Collett et al., *Virology* 144:228-245, 1985)), Paramyxoviridae (e.g., Newcastle disease virus (Millar et al., *J. Gen. Virol.* 69:613-620, 1988; Chambers et al., *Nucl. Acid. Res.* 14:9051-9061, 1986; Schaper et al., *Virology* 165:291-295, 1988), and canine distemper virus (Curran et al., *J. Gen. Virol.* 72:443-447, 1991; Barrett et al., *Virus Res.* 8:373-386, 1987; Bellini et al., *J. Virol.* 58:408-416, 1986)), Togaviridae (e.g., WEE virus (Weaver et al., *Virology* 197:375-390, 1993), EEE virus (Chang et al., *J. Gen. Virol.* 68:2129-2142, 1987), and VEE virus (Kinney et al., *Virology* 152:400-413, 1986)), Rhabdoviridae (e.g., vesicular stomatitis virus (Gill et al., *Virology* 150:308-312, 1986; Gallione et al., *J. Virol.* 46:162-169, 1983; Banerjee et al., *Virology* 137:432-438, 1984), and rabies virus (Tordo et al., *Nucl. Acid. Res.* 14:2671-2683, 1986; Hiramatsu et al., *Virus Genes* 7:83-88, 1993; Kieny et al., *Nature* 312:163-166, 1984)), Coronaviridae (e.g., transmissible gastroenteritis virus (Britton et al., *Molec. Micro.* 2:89-99, 1988; Godet et al., *Virology* 188:166-175, 1992; Jackwood et al., *Adv. Exp. Med. and Biol.* 342:43-48, 1993), and feline infectious peritonitis virus (Reed et al., *Adv. Exp. Med. and Biol.* 342:17-21, 1993)), Reoviridae (e.g., porcine rotavirus (Burke et al., *J. Gen. Virol.* 75:2205-2212, 1994; Nishikawa et al., *Nucl. Acid. Res.* 16:11847, 1988)), Orthomyxoviridae (e.g. equine influenza (Gibson et al., *Virus Res.* 22:93-106, 1992; Dale et al., *Virology* 155:460-468, 1986)), Picomaviridae (e.g., FMD virus (Graham et al., *Virology* 176:524-530, 1990; Brown et al., *Gene* 75:225-233, 1989; Fross et al., *Nucl. Acid. Res.* 12:6587-6601, 1984)), and Herpesviridae (e.g., equine herpesvirus (Crabb et al., *J. Gen. Virol.* 72:2075-2082)).

In other embodiments, the sequences which encode immunoreactive polypeptides of the pathogenic agents may be chosen from a group that includes the agents of coccidiosis (e.g., *Eimeria Acervulina*, *E. tenella*, *E. maxima* (Talebi et al., *Infect. Immun.* 62:4202-4207, 1994; Pasamotites et al., *Mol. Biochem. Parasit.* 57:171-174, 1993; Tomley et al., *Mol. Biochem. Parasit.* 49:277-288, 1991; Castle et al., *J. of Parasit.* 77:384-390, 1991; Jenkins et al., *Exp. Parasit.* 70:353-362, 1990)), anaplasmosis (e.g., *Anaplasma marginale* (McGuire et al., *Vaccine* 12:465-471, 1994; Palmer et al., *Infect. Immun.* 62:3808-3816, 1994; Oberle et al., *Gene* 136:291-294, 1993; Barbet et al., *Infect. Immun.* 59:971-976, 1991; Barbet et al., *Infect. Immun.* 55:2428-2435, 1987)), babesiosis (e.g., *Babesia bovis* (Suarez et al., *Infect. Immun.* 61:3511-3517, 1993; Hines et al., *Mol. Biochem. Parasit.* 55:85-94, 1992; Jamer et al., *Mol. Biochem. Parasit.* 55:75-83, 1992; Suarez et al., *Mol. Biochem. Parasit.* 46:45-52, 1991)), theileriosis (e.g., *Theileria parva* (Nene et al., *Mol. Biochem. Parasit.* 51:17-27, 1992; Iams et al., *Mol. Biochem. Parasit.*

39:47-60, 1990)), malaria (e.g. *Plasmodium falciparum* (Haeseleer et al., *Mol. Biochem. Parasit.* 57:117-126, 1993)), salmonellosis (*Salmonella typhimurium* and *S. dublin*), bovine and ovine mastitis (*Staphylococcus aureus*), bovine tuberculosis (*Mycobacterium bovis*), pseudotuberculosis (*Yersinia pseudotuberculosis*), coccidioidomycosis (*Coccidioides immitis*), cryptococcosis (*Cryptococcus neoformans*), anthrax (*Bacillus anthracis*), brucellosis (*Brucella abortus* and *B.suis*), and leptospirosis (*Leptospira interrogans* and *L.biflexa*).

To illustrate this aspect in more detail, methods used in constructing recombinant alphavirus vectors and eukaryotic layered vector initiation systems containing these sequences for veterinary application are described for two of the above pathogenic agents (one viral and one parasitic). The construction of additional alphavirus vectors and eukaryotic layered vector initiation systems is readily accomplished by those skilled in the art, based on the following methodologies and using sequences from other related or non-related pathogenic agents. In the case of foot-and-mouth disease virus (FMDV), a cassette comprising each of the four P1 capsid proteins (1A, 1B, 1C, 1D) and the 3C protease responsible for their post-translational cleavage is obtained as plasmids MR1 or MR2 from Graham et al. (*Virology* 176:524-530, 1990). Plasmid MR1 or MR2 is digested with the enzymes HindIII and DraI to remove the FMDV P1 cassette, followed by fill-in of the HindIII terminus with Klenow, and purification from a 1% agarose gel using GENECLEAN™. Plasmid vectors pKSSINBV and pVGELVIS-SINBV (see Example 3) are digested with XhoI and the termini also made blunt using Klenow, followed by treatment with CIAP and purification from a 1% agarose gel using GENECLEAN™. The purified fragments are subsequently ligated to generate the alphavirus vector construct pKSSIN-FMDV and eukaryotic layered vector initiation system plasmid pVGELVIS-FMDV. The purified FMDV sequences are also readily inserted into any of the other vector constructs described in this invention (see Example 3). Packaging of the FMDV-containing alphavirus vector construct pKSSIN-FMDV can be accomplished as described in Example 7.

For construction of a recombinant alphavirus vector construct or eukaryotic layered vector initiation system comprising sequences from a pathogenic agent of anaplasmosis, the major surface protein 2 (MSP-2) of *A. marginale* is obtained by PCR amplification from plasmid pCKR11.2 (Palmer et al., *Infect. Immun.* 62:3808-3816, 1994) using the following oligonucleotide pair, each containing a flanking XhoI site:

forward primer (AM-MSP-2F):

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5'-TATATCTCGAGACCACCATGAGTGCTGTAAGTAATAGGAAGC  
(SEQ. I.D. NO. 115)

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reverse primer (AM-MSP-2R):

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5'-TATATCTCGAGCTAGAAGGCAAACCTAACACCCAAC  
(SEQ. I.D. NO. 116)

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A standard three temperature cycling protocol is performed as described previously using THERMALASE™ thermostable polymerase, the oligonucleotide pair, and plasmid pCKR11.2 as template. Following amplification, the MSP-2 amplicon is purified using GENECLEAN™, digested with XhoI, and re-purified with GENECLEAN™. Plasmid vectors pKSSINBV and pVGELVIS-SINBV (see Example 3)

also are digested with XhoI, followed by treatment with CIAP and subsequent ligation to the MSP-2 fragment to generate the alphavirus vector construct pKSSIN-MSP2 and eukaryotic layered vector initiation system plasmid pVGELVIS-MSP2. The purified MSP-2 sequences are also readily inserted into any of the other vector constructs described elsewhere in this specification (e.g., Example 3). Packaging of the MSP-2-containing alphavirus vector construct pKSSIN-MSP2 can be accomplished as described in Example 7.

#### Example 4

##### A. INSERTION OF ADENOVIRUS EARLY REGION E3 GENE INTO SINDBIS VECTORS

In order to inhibit the host CTL response directed against viral specific proteins expressed in vector infected cells, in applications where repeated administration of the therapeutic is desired, the Adenovirus type 2 (Ad 2) E3/19K gene ATCC No. VR-846 is cloned into the pKSSINd1JRsirc plasmid, immediately downstream from the junction region core. Briefly, Ad 2 is propagated in a permissive cell line, for example HeLa or Vero cells, and after evidence of cytopathologic effects, virions are purified from the cell lysate, and the Ad 2 DNA is purified from the virus.

The Ad 2 DNA E3/19K gene, including the amino terminal signal sequence, followed by the intraluminal domain and carboxy terminal cytoplasmic tail which allows the E3 19K protein to embed itself in the endoplasmic reticulum, is located between viral nucleotides 28,812 and 29,288. Isolation of the Ad 2 E3 19K gene from the viral genomic DNA is accomplished by PCR amplification, with the primer pair shown below:

Ad 2 E3 Forward primer (Ad 2 nucleotides 28,812–28,835):

5'-TAT ATC TCC AGA TGA GGT ACA TGA TTT TAG GCT TG-3'  
(SEQ. ID NO. 56)

Ad 2 E3 Reverse primer (Ad 2 nucleotides 29,241–29,213):

5'-TAT ATA TCG ATT CAA GGC ATT TTC TTT TCA TCA ATA AAA C  
(SEQ. ID NO. 57)

In addition to the Ad 2 complementary sequences, both primers contain a five nucleotide 'buffer sequence' at their 5' ends for efficient enzyme digestion of the PCR amplicon products. This sequence in the forward primer is followed by the Xho I recognition site, and in the reverse primer this sequence is followed by the Cla I recognition site. Thus, in the 5' to 3' direction, the E3/19K gene is flanked by Xho I and Cla I recognition sites. Amplification of the E3/19K gene from Ad 2 DNA is accomplished with the following PCR cycle protocol:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.17	5
72	3.5	
94	0.5	30
70	3.5	
72	10	10

Following amplification, the 451 bp amplicon is purified on a 1.5% agarose gel, and digested with the Xho I and Cla I enzymes. pKSSINd1JRsirc plasmid is partially digested

with ClaI. Plasmid that has been digested only once is isolated by gel electrophoresis then digested with XhoI. The large fragment is isolated by gel electrophoresis and ligated to the digested PCR amplicon. This clone is designated pKSSINd1JRsircAdE3. Using the same cloning strategy, the Ad 2 E3/19K gene may be inserted into any of the modified synthetic junction region vectors or ELVIS vectors described in Example 3.

##### B. INSERTION OF THE HUMAN CYTOMEGALOVIRUS H301 GENE INTO SINDBIS VECTORS

In order to inhibit the host CTL directed response against viral specific proteins expressed in vector infected cells in applications where repeated administration of the therapeutic is desired, the human cytomegalovirus (HCMV) H301 gene is cloned into the pKSSINd1JRsirc plasmid, immediately downstream from the junction region core.

Briefly, HCMV strain AD169 (ATCC No. VR-538), is propagated in a permissive cell line, for example primary human foreskin fibroblasts (HFF) (GIBCO/BRL, Gaithersburg, Md.), and after evidence of cytopathologic effects, virions are purified from the cell lysate. Subsequently, HCMV DNA is purified from the virions.

The HCMV H301 gene is located between viral nucleotides 23,637 and 24,742. Isolation of the HCMV H301 gene from the viral genomic DNA is accomplished by PCR amplification, with the primer pair shown below:

HCMV H301 Forward primer (buffer sequence/Xho I site/  
HCMV nucleotides 23,637–23,660):

5'-TAT ATC TCC AGA TGA TGA CAA TGT GGT GTC TGA CG-3'  
(SEQ. ID NO. 58)

HCMV H301 Reverse primer (buffer sequence/Cla I site/  
HCMV nucleotides 24,744–24,722):

5'-TAT ATA TCG ATT CAT GAC GAC CGG ACC TTG CG-3'  
(SEQ. ID NO. 59)

In addition to the HCMV H301 gene complementary sequences, both primers contain a five nucleotide 'buffer sequence' at their 5' ends for efficient enzyme digestion of the PCR amplicon products. This sequence in the forward primer is followed by the Xho I recognition site, and in the reverse primer this sequence is followed by the Cla I recognition site. Thus, in the 5' to 3' direction, the HCMV H301 gene is flanked by Xho I and Cla I recognition sites. Amplification of the HCMV H301 gene from HCMV DNA is accomplished with the following PCR cycle protocol:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.17	5
72	3.5	
94	0.5	30
70	3.5	
72	10	10

Following amplification, the 1,129 bp amplicon product is purified on a 1.0% agarose gel, and subsequently digested with the Xho I and Cla I enzymes and ligated into the CIAP treated pKSSINd1JRsirc plasmid, previously digested with Xho I and Cla I as described above. This clone is designated pKSSINd1JRsircH301. Using the same cloning strategy, the HCMV H301 gene is inserted into all of the modified

synthetic junction region vectors and all of the ELVIS vectors described in Example 3.

#### Example 5

#### EXPRESSION OF MULTIPLE HETEROLOGOUS GENES FROM SINDBIS VECTORS

The plasmid pBS-ECAT (Jang et al., *J. Virol* 63:1651, 1989) includes the 5' nontranslated region of Encephalomyocarditis virus (EMCV) from nts 260-848 of the viral genome, which contains the internal ribosome entry site (IRES). EMCV nucleotides 260-827 are amplified from pBS-ECAT by PCR, using the following primer pair. EMCV IRES Forward primer A (For insertion next to disabled junction region in vector pKSSINBVd1JR at Apa I site):

5'-TAT ATG GGC CCC CCC CCC CCC AAC G-3'  
(SEQ. ID NO. 60)

EMCV IRES Forward primer B (For insertion between heterologous genes terminating with Cla I sites and initiating with Nco I sites):

5'-TAT ATA TCG ATC CCC CCC CCC CCC CCA ACG-3'  
(SEQ. ID NO. 61)

EMCV IRES Reverse Primer (To be used with either primers A or B):

5'-TAT ATC CAT GGC TTA CAA TGG TTT TCA AAG G-3'  
(SEQ. ID NO. 62)

The amplicon resulting from amplification with the forward primer A and the reverse primer is flanked by Apa I and Nco I recognition sites, inside a 5 bp 'buffer sequence'. The amplicon resulting from amplification with the forward primer B and the reverse primer is flanked by Cla I and Nco I recognition sites, inside a 5 bp 'buffer sequence'. Amplification of the EMCV IRES sequence from the pBS-ECAT plasmid is accomplished with the following PCR cycle protocol:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.17	5
72	3.5	
94	0.5	30
70	3.5	
72	10	1

In a similar manner, the ATG corresponding to the start codon of the heterologous gene to be inserted immediately downstream of the EMCV IRES insert is modified to contain an Nco I site (CCATGG) while the 3' end is modified to contain a Cla I site.

For insertion into the pKSSINBVd1JR vector, the 589 bp EMCV-IRES amplicon is digested with Apa I and Nco I, purified on a 1% agarose gel. The heterologous gene amplicon is digested with Nco I and Cla I and purified in a similar manner. Both fragments are ligated into the CIAP treated vector digested with Apa I and Cla I as described in example 4.

For insertion into the pKSSINBV or pKSSINBVd1JRsirc vectors between heterologous genes, the 589 bp amplicon is digested with Cla I and Nco I, purified on a 1% agarose gel, and ligated into the bicistronic heterologous gene vector digested with Cla I and Nco I and treated with CIAP. In a bicistronic heterologous gene configuration, the 3' end of the upstream heterologous gene is modified to terminate in a Cla I recognition site. The ATG corresponding to the start codon of the second downstream heterologous gene to be inserted immediately downstream of the EMCV IRES insert is modified to contain an Nco I site (CCATGG). Thus, from 5' to 3', the order of components is: pKSSINBV or pKSSINBVd1JRsirc-gene #1-Cla/Nco EMCV IRES gene #2-3' SIN. Insertion into all of the modified junction region vectors described in Example 2 and all of the ELVIS vectors described in Example 3 follows the strategy given here for the pKSSINBV or pKSSINBVd1JRsirc vectors.

The pKSSINBVd1JR vector containing a bicistronic heterologous configuration is constructed with each of the EMCV IRES amplicons described above. The first EMCV IRES amplicon is flanked by Apa I and Nco I sites and is inserted immediately downstream of the disabled junction region at the Apa I site, as described above. This EMCV IRES sequence is followed by the first heterologous gene, which terminates in a Cla I recognition site. The first heterologous gene is followed by the second EMCV IRES sequence, using the amplicon flanked by Cla I and Nco I recognition sites. The second heterologous gene follows the second EMCV IRES sequence. Thus, from 5' to 3', the order of components is: SINBVd1JR-Apa/Nco EMCV IRES gene #1-Cla/Nco EMCV IRES gene #2-3' SIN.

The plasmid pP2-5' (Pelletier et al., *Mol. Cell Biol.* 8:1103, 1988) includes the 5' nontranslated region of the poliovirus P2/Lansing strain from nucleotides 1-1,872 of the viral genome, which contains the polio IRES. Poliovirus nucleotides 320-631 are amplified from pP2-5' by PCR, using the following primer pair: Polio IRES Forward primer A (For insertion next to disabled junction region in vector pKSSINBVd1JR at Apa I site):

5'-TAT ATG GGC CCT CGA TGA GTC TGG ACG TTC CTC-3'  
(SEQ. ID NO. 63)

Polio IRES Forward primer B (For insertion between heterologous genes terminating with Cla I sites and initiating with Nco I sites):

5'-TAT ATA TCG ATT CGA TGA GTC TGG ACG TTC CTC-3'  
(SEQ. ID NO. 64)

Polio IRES Reverse Primer (To be used with either primers A or B):

5'-TAT ATC CAT GGA TCC AAT TTG CTT TAT GAT AAC AAT C-3'  
(SEQ. ID NO. 65)

The amplicon resulting from PCR with the Polio IRES forward primer A/reverse primer pair shown above is flanked by Apa I and Nco I recognition sites, inside a 5 bp 'buffer sequence'. The amplicon resulting from PCR with the Polio IRES forward primer B/reverse primer pair is shown above is flanked by Cla I and Nco I recognition sites, inside a 5 bp 'buffer sequence'. Amplification of the polio IRES sequence from the pP2-5' plasmid is accomplished with the PCR protocol shown in Example 5. In a similar

manner, the ATG corresponding to the start codon of the heterologous gene to be inserted immediately downstream of the Polio IRES insert is modified to contain an NcoI site (CCATGG) while the 3' end is modified to contain a ClaI site.

For insertion into the pKSSINBVd1JR vector, the 333 bp Polio-IRES amplicon is digested with Apa I and Nco I and purified on a 1.5% agarose gel. The heterologous gene amplicon is digested with NcoI and ClaI and purified in a similar manner. Both fragments are ligated into the CIAP treated vector digested with Apa I and ClaI as described in example 4.

For insertion into the pKSSINBV or pKSSINBVd1JRsirc vectors between heterologous genes, the 333 bp amplicon is digested with Cla I and Nco I, purified on a 1.5% agarose gel, and ligated into the bicistronic heterologous gene vector digested with Cla I and Nco I and treated with CIAP. In a bicistronic heterologous gene configuration, the 3' end of the upstream heterologous gene is modified to terminate in a Cla I recognition site. The ATG corresponding to the start codon of the second downstream heterologous gene to be inserted immediately downstream of the polio IRES insert is modified to contain an Nco I site (CCATGG). Thus, from 5' to 3', the order of components is: pKSSINBV or pKSSINBVd1JRsirc-gene #1-Cla/Nco polio IRES gene #2-3' SIN. Insertion into all of the modified junction region vectors and all of the ELVIS vectors described in Example 3 follows the strategy given here for the pKSSINBV or pKSSINBVd1JRsirc vectors.

The pKSSINBVd1JR vector containing a bicistronic heterologous configuration is constructed with each of the polio IRES amplicons described above. The first polio IRES amplicon is flanked by Apa I and Nco I sites and is inserted immediately downstream of the disabled junction region at the Apa I site, as described above. This polio IRES sequence is followed by the first heterologous gene, which terminates in a Cla I recognition site. The first heterologous gene is followed by the second polio IRES sequence, using the amplicon flanked by Cla I and Nco I recognition sites. The second heterologous gene follows the second polio IRES sequence. Thus, from 5' to 3', the order of components is: SINBVd1JR-Apa/Nco polio IRES gene #1-Cla/Nco EMCV IRES gene #2-3' SIN.

The 220 bp BiP cDNA, corresponding to the 5' leader region of the human immunoglobulin heavy-chain binding protein mRNA, is amplified from a plasmid containing the 5' noncoding region of the BiP gene, pGEM5ZBiP5' (provided by P. Sarnow, University of Colorado Health Sciences Center), using PCR. The sequence corresponding to BiP cDNA was determined originally in the bacteriophage lambda hu28-1 clone of the human GRP78 gene (Ting and Lee, *DNA* 7:275-286, 1988). The forward primer to be used in the PCR reaction varies, depending on the Sindbis vector into which the BiP cDNA is inserted. The reverse primer for the PCR reaction is the same for all Sindbis vectors. Amplification of the BiP cDNA sequence from pGEM5ZBiP5' from the plasmid for insertion into the Sindbis vector pKSSINBVd1JR, immediately downstream of the disabled junction region, is accomplished by amplification with the following forward primer:

---

5'-TAT ATG GGC CCG GTC GAC GCC GGC CAA GAC-3'  
(SEQ. ID NO. 66)

---

In addition to the BiP cDNA complementary sequences, beginning at nucleotide 12, the primer contains a five nucleotide 'buffer sequence' at its 5' end for efficient enzyme

digestion of the PCR amplicon products. This sequence is followed by the Apa I recognition site.

Amplification of the BiP cDNA sequence from the pGEM5ZBiP5' plasmid for insertion into the Sindbis vectors pKSSINBV, or pKSSINBVd1JRsirc, is accomplished by amplification with the following forward primer shown below. For these vectors, the BiP cDNA is inserted between two heterologous genes, which are placed in the region corresponding to the Sindbis structural genes.

---

5'-TAT ATA TCG ATG GTC GAC GCC GGC CAA GAC-3'  
(SEQ. ID NO. 67)

---

In addition to the BiP cDNA complementary sequences, beginning at nucleotide 12, the primer contains a five nucleotide 'buffer sequence' at its 5' end for efficient enzyme digestion of the PCR amplicon products. This sequence is followed by the Cla I recognition site.

The reverse primer for amplification of the BiP cDNA sequence from the pGEM5ZBiP5' plasmid for insertion into the Sindbis vectors pKSSINBVd1JR, pKSSINBV, or pKSSINBVd1JRsirc, is:

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5'-TAT ATC CAT GGT GCC AGC CAG TTG GGC AGC AG-3'  
(SEQ. ID NO. 68)

---

In addition to the BiP cDNA complementary sequences, beginning at nucleotide 12, the reverse primer contains a five nucleotide 'buffer sequence' at its 5' end for efficient enzyme digestion of the PCR amplicon products. This sequence is followed by the Nco I recognition site. Amplification of the BiP cDNA from the pGEM5ZBiP5' is accomplished with PCR protocol that are described above. In a similar manner, the ATG corresponding to the start codon of the heterologous gene to be inserted immediately downstream of the BiP IRES insert is modified to contain an NcoI site (CCATGG) while the 3' end is modified to contain a ClaI site.

For insertion into the pKSSINBVd1JR vector, the 242 bp BiP IRES amplicon is digested with Apa I and Nco I and purified on a 2% agarose gel. The heterologous gene amplicon is digested with NcoI and ClaI and purified in a similar manner. Both fragments are ligated into the CIAP treated vector digested with Apa I and ClaI as described in example 4.

For insertion into the pKSSINBV or pKSSINBVd1JRsirc vectors between heterologous genes, the 242 bp BiP IRES amplicon is digested with Cla I and Nco I, purified on a 2% agarose gel, and ligated into the bicistronic heterologous gene vector digested with Cla I and Nco I and treated with CIAP. In a bicistronic heterologous gene configuration, the 3' end of the upstream heterologous gene is modified to terminate in a Cla I recognition site. The ATG corresponding to the start codon of the second downstream heterologous gene to be inserted immediately downstream of the BiP cDNA insert is modified to contain an Nco I site (CCATGG). Thus, from 5' to 3', the order of components is: pKSSINBV or pKSSINBVd1JRsirc-gene #1-Cla/Nco BiP-gene #2-3' SIN. Insertion into all of the modified junction region vectors described in Example 2, and into all of the ELVIS vectors described in example 3, follows the strategy given here for the pKSSINBV or pKSSINBVd1JRsirc vectors.

The pKSSINBVd1JR vector containing a bicistronic heterologous configuration is constructed with each of the BiP cDNA amplicons described above. The first BiP cDNA amplicon is flanked by Apa I and Nco I sites and is inserted immediately downstream of the disabled junction region at



the Apa I site, as described above. This BiP sequence is followed by the first heterologous gene, which terminates in a Cla I recognition site. The first heterologous gene is followed by the second BiP cDNA sequence, using the amplicon flanked by Cla I and Nco I recognition sites. The second heterologous gene follows the second BiP sequence. Thus, from 5' to 3', the order of components is: SINBVd1JR-Apa/Nco BiP-gene #1-Cla/Nco BiP-gene #2-3' SIN.

Sequences which promote ribosomal readthrough are placed immediately downstream of the disabled junction region in the pKSSINBVd1JR vector, which allows ribosomal scanning in genomic mRNA from non-structural gene termination to the heterologous genes. The heterologous proteins are expressed from genomic length mRNA by ribosomal scanning. This extends the life of the infected target cell because no subgenomic transcription occurs in cells infected with this vector. Further, these same ribosomal scanning sequences are placed between heterologous genes contained in polycistronic subgenomic mRNAs. The ribosomal spanning sequence to be used in the pKSDINBVd1JR vector and between heterologous genes in the polycistronic mRNA region is:

5'-TTA ATT AAC GGC CGC CAC CAT GG-3' (SEQ. ID NO. 69)

The boldfaced codons refer to the ochre stop codon and AUG start codon, respectively. The bases underlined surrounding the stop codon refer to the Pac I recognition site and the bases underlined surrounding the start codon refer to the Nco I recognition site. The intercistronic distance of 15 bp between the start and stop codons allows efficient ribosomal readthrough, as shown previously (Levine et al., *Gene* 108:167-174, 1991). The sequences surrounding the ATG start codon from bases -9 to +1 conform to the Kozak consensus sequence for efficient translational initiation (Kozak, *Cell* 44:283-292, 1986). Where possible, the 3' terminal nucleotide corresponding to the carboxy terminal amino acid is changed to T, by site-directed mutagenesis. Also, the 5' terminal nucleotide corresponding to the amino terminal amino acid in the downstream cistron is changed to G, by site-directed mutagenesis.

Insertion of the intercistronic sequence between heterologous genes, or downstream of the disabled junction region in vector pKSDINBVd1JR, modified as described above, is accomplished by insertion of the double-stranded oligonucleotide pair shown below, into compatible Pac I/Nco I ends:

Read through sense Oligonucleotide:

5'-TAA CGG CCG CCA C-3' (SEQ. ID NO. 70)

Read through antisense Oligonucleotide:

5'-CCA TGG TGG CGG CCG TTA AT-3' (SEQ. ID NO. 71)

The oligonucleotides above are mixed in equal molar quantities in the presence of 10 mM MgCl<sub>2</sub>, heated at 95° C. for 5 min, then allowed to cool slowly to room temperature, yielding the desired intercistronic sequence flanked by Pac I and Nco I sites. The intercistronic sequence is then ligated into the appropriate vector containing Pac I and Nco I compatible sites.

Another aspect of the present invention to enable expression of multiple heterologous genes in eukaryotic layered vector initiation systems is based on the use of alternate splicing signals. In this configuration, a splice donor sequence is inserted immediately downstream of the junction region promoter, followed by one or more heterologous

genes, each of which is preceded by a splice acceptor sequence. As such, multiple splice acceptor/heterologous gene inserts may be arrayed 3' to one another. This creates a system whereby multiple heterologous genes are expressed from a single eukaryotic layered vector initiation system transcript, which is processed alternately at each splice acceptor site to give rise to individual autocatalytic RNAs encoding an individual heterologous gene. In such a system, levels of expression for each heterologous gene is controlled independently by altering the nucleotide sequence of the splice acceptor site. In addition, multiple splice donor/acceptor sites may be engineered into the system. Finally, tissue specific splice donor/acceptor sequences may be utilized in such a system to control the expression in specific tissues.

#### Example 6

##### EXPRESSION OF MULTIPLE HETEROLOGOUS GENES BY COPACKAGING

The ability to copackage multiple RNA molecules in the same alphavirus vector particle can be useful for the expression of multiple heterologous gene products from a single alphavirus vector particle. In addition, this concept can also be adapted in order to allow very large genes to be carried on RNA molecules separate from the alphavirus vector RNA containing the nonstructural genes, thus avoiding the need to package very long vector RNA molecules.

In order to accomplish such copackaging, all RNA fragments must contain a 5' sequence which is capable of initiating transcription of an alphavirus RNA, an alphavirus RNA polymerase recognition sequence for minus-strand synthesis, and at least one copy of the RNA packaging sequence. At least one of the RNA fragments also must contain sequences which code for the alphavirus non-structural proteins. Within preferred embodiments of the invention, one or more of the RNA fragments to be copackaged also will contain a viral junction region followed by a heterologous gene.

##### A. CONSTRUCTION OF COPACKAGED EXPRESSION CASSETTES FOR EXPRESSION OF MULTIPLE HETEROLOGOUS GENES

In order to demonstrate the feasibility of copackaging to allow for the expression of multiple heterologous genes, two vector constructs are created. The first construct consists of a 5' sequence that is capable of initiating transcription of Sindbis virus RNA, Sindbis RNA sequences required for packaging, sequences encoding the synthesis of nonstructural proteins 1-4, a Sindbis junction region, the luciferase gene, and Sindbis 3' sequences required for synthesis of the minus strand RNA. The second construct consists of a 5' sequence that is capable of initiating transcription of a Sindbis virus, Sindbis sequences required for packaging, a Sindbis Junction region, Sequences encoding the LacZ gene, and Sindbis 3' sequences required for synthesis of the minus strand RNA. RNA transcripts of these constructs transfected into a packaging cell line are copackaged to produce a vector particle capable of transferring expression of both luciferase and  $\beta$ -galactosidase into the same eukaryotic cell.

The  $\beta$ -galactosidase reporter gene is inserted into the Sindbis Basic Vector (pKSSINBV) followed by deletion of a portion of the Sindbis non-structural proteins from the vector. RNA from this construct is cotransfected with RNA from Sindbis Luciferase Vector (pKSSINBV-luc) and is copackaged by one of the methods described in Example 7. Infection of fresh BHK-21 cells with vector particles containing the copackaged RNA expression cassettes should

result in the expression of both luciferase and  $\beta$ -galactosidase in the same cell.

#### B. CONSTRUCTION OF A $\beta$ -GALACTOSIDASE EXPRESSION CASSETTE

pKSSINBV-Linker is digested with the enzyme Sac I, which cleaves immediately after the Sindbis 3'-end and poly A sequence. The digested fragment is treated with alkaline phosphatase and purified using GeneClean. Two 12 mer oligonucleotides,

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5' GGTTTAAACAGGAGCT 3' (SEQ. ID NO. 72)  
5' CCTGTTAAACAGGAGCT 3' (SEQ. ID NO. 73)

---

which form the Pme I site with SacI compatible ends when hybridized, were phosphorylated and ligated into the SacI digested vector. This construct is known as pKSSINBV-Linker-PmeI. The Pme I recognition site is substituted for the Sac I site in order to create a site for linearization of the plasmid prior to SP6 transcription. The lacZ gene contains several Sac I sites. pKSSINBV-Linker-PmeI is digested with Pml I and Bcl I followed by purification with GENECLAN. The lacZ gene is obtained by digestion of pSV  $\beta$ -galactosidase vector DNA (Promega Corp., Madison, Wis.) with the enzyme HindIII. The digest is blunt-ended with Klenow DNA polymerase and dNTPs. The Klenow is heat killed and the plasmid is further digested with Bam HI and Xmn I. Xmn I reduces the size of the remaining vector fragment to simplify gel purification of the lacZ fragment. The 3.7 kbp lacZ fragment is purified from a 1% agarose gel and ligated into the Pml I/Bcl I digested pKSSINBV-Linker-PmeI fragment. This construct is known as pKSSINBV-lacZ. pKSSINBV-lacZ is digested with Bsp EI and religated under dilute conditions. This results in the removal of the Sindbis nonstructural proteins between nt#422-7054. This Sindbis construct is known as pKSSINBVd1NSP-lacZ.

pKSSINBVd1NSP-lacZ and pKSSINBV-luc are linearized with Pme I and Sac I, respectively, and SP6 transcripts are prepared as described in Example 3. These RNA transcripts are cotransfected into packaging cells that express the Sindbis structural proteins by one of the mechanisms described in Example 7. Each RNA transcript contains a 5' sequence that is capable of initiating transcription of a Sindbis virus, RNA sequences required for packaging, a Sindbis junction region, a reporter gene, and Sindbis 3' sequences required for synthesis of the minus strand RNA. The pKSSINBV-luc transcript also contains the Sindbis non-structural proteins. In cotransfected cells, both RNA transcripts are replicated and some viral particles will contain both RNA transcripts copackaged into the same particle. Infection of fresh cells with the copackaged RNA particles will result in cell that express both luciferase and  $\beta$ -galactosidase.

#### C. COPACKAGING OF MULTIPLE EXPRESSION CASSETTES TO INCREASE PACKAGING CAPACITY

Large genes such as Factor VIII can benefit from copackaging. Briefly, insertion of the cDNA coding for Factor VIII into the Sindbis Basic Vector (PKSSINBV) results in an RNA transcript approaching 16 kb in length. Because of the increased length, this RNA cannot be replicated or packaged efficiently. Using approaches described above, the Sindbis nonstructural proteins and the Factor VIII gene could be divided onto separate RNA molecules of approximately 8 kb and 9 kb in length, and copackaged into the same particles.

#### D. CONSTRUCTION OF A FACTOR VIII EXPRESSION CASSETTE

The pKSSINBV-Linker-PmeI construct is digested with the enzyme Bsp EI and religated under dilute conditions.

This results in the removal of the Sindbis nonstructural proteins between nt# 422-7054. This construct is known as pKSSINBVd1NSP-Linker-PmeI. The pKSSINBVd1NSP-Linker-PmeI construct is digested with the enzymes Pml I and Stu I and purified by using GeneClean. The source of Factor VIII cDNA is clone pSP64-VIII, an ATCC clone under the accession number 39812 having a cDNA encoding the full-length human protein. pSP64-VIII is digested with Sal I, the ends are blunted with T4 DNA polymerase and 50  $\mu$ M of each dNTP, and the ca. 7700 bp. fragment is electrophoresed on a 0.7% agarose/TBE gel and purified with GeneClean. The 7.7 kb fragment encoding Factor VIII is purified in a 0.7% agarose gel and subsequently ligated to the Pml I/Stu I digested pKSSINBVd1NSP-Linker-PmeI fragment. This construct is known as pKSSINBVd1NSP-Factor VIII.

pKSSINBVd1NSP-Factor VIII and pKSSINBV constructs are linearized with Pme I and Sac I, respectively. SP6 transcripts are prepared as described in Example 3. These RNA transcripts are cotransfected into packaging cells that express the Sindbis structural proteins by one of the mechanisms described in Example 7. Both RNA transcripts contain a 5' sequence that is capable of initiating transcription of Sindbis RNA, sequences required for RNA packaging, a Sindbis Junction region, and the Sindbis 3' sequences required for synthesis of the minus strand RNA. In addition, the pKSSINBV transcript contains the Sindbis nonstructural protein genes, and the pKSSINBVd1NSP-Factor VIII construct contains the Factor VIII gene, but not the Sindbis nonstructural protein genes. In cotransfected cells, both RNA transcripts are replicated and some viral particles will contain both RNA transcripts copackaged into the same vector particle. Infection of fresh BHK-21 cells with the copackaged RNA will result in Factor VIII expression only if both RNA molecules are present in the same cell.

#### E. CONSTRUCTION OF AN AURA VIRUS COPACKAGING VECTOR

To develop Aura virus expression systems analogous to those described for Sindbis, standard techniques known in the art (e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989), as well as specific approaches described herein, will be utilized for constructions. Virus, obtained from the ATCC, is propagated on cultured cells, its virion RNA extracted, and cDNA spanning the entire genome synthesized and cloned using conventional techniques. This cDNA is then used to construct gene transfer vector systems similar in principal to those described above, including, but not limited to, a replicon capable of carrying the heterologous gene(s), packaging cell lines that express the structural protein genes, and unique to this system, a separate packaging-competent subgenomic vector capable of carrying the additional heterologous gene(s). Since Aura virus subgenomic RNA contains a packaging signal, preliminary experiments are performed to identify this sequence, in order to prevent its inactivation during replacements with heterologous the gene(s). After identification of the packaging sequence, the individual elements of this Aura-based system are generated.

A basic replicon vector is constructed to contain the following minimum elements: Aura 5' sequences necessary for replication, nonstructural protein coding regions, a modified or unmodified junction region for subgenomic mRNA synthesis, a restriction enzyme site for insertion of heterologous gene(s), one or more copies of the packaging signal, and 3' Aura sequences necessary for replication, including a polyadenylate sequence. An upstream bacteriophage RNA

polymerase promoter will be utilized for in vitro transcription of replicon RNA; alternatively, a eukaryotic RNA polymerase promoter will be utilized for transcription directly from cDNA.

A packaging-competent subgenomic vector is also constructed to contain the following minimum elements: a modified or unmodified junction region, a restriction enzyme site for insertion of heterologous gene(s), one or more copies of the packaging signal, and 3' Aura sequences necessary for replication/minus-strand synthesis, including a polyadenylate sequence. The subgenomic vector may, in some cases, be constructed with the Aura 5' replication sequences positioned upstream of the junction region, such that the vector will function as an amplicon. Transcription of subgenomic vector RNA can be accomplished in vitro using a bacteriophage RNA polymerase promoter, or cDNA in vivo using a eukaryotic RNA polymerase promoter. Further, the initial transcript may be of the sense-configuration or of the antisense-configuration.

Packaging cell lines are also constructed as described previously for Sindbis vectors, such that mRNA for one or more of the structural proteins will be transcribed from the junction region and be inducible by the Aura replicon. In other cases, one or more of the structural proteins can be expressed under the control of an inducible or constitutive eukaryotic promoter. In each case, specific inactivating mutations are made in any packaging sequences present in the structural protein genes, in order to prevent encapsidation of these sequences with the replicon. These mutations should be silent changes, usually at the third position of the codon, which do not affect the amino acid encoded.

The ability to package multiple heterologous genes can be exploited for many therapeutic applications, which include, but are not limited to, expression of multiple cytokines, multiple CTL epitopes, combinations of cytokines and CTL epitopes to enhance immune presentation, multiple subunits of a therapeutic protein, combinations of therapeutic proteins and antisense RNAs, etc. In addition to its utility for the expression of multiple heterologous genes, the packaging of subgenomic mRNAs into virions also enables this vector system for the transfer of extremely long heterologous sequences. Furthermore, this multipartite approach is useful in the development of producer cell lines, wherein replicase proteins and structural proteins are being stably expressed, and any heterologous gene contained within a subgenomic vector could then be readily introduced as a stable integrant.

#### Example 7

#### CONSTRUCTION OF ALPHAVIRUS PACKAGING CELL LINES

##### A. SELECTION OF PARENT CELL LINES FOR ALPHAVIRUS PACKAGING CELL LINE DEVELOPMENT

##### 1. PERSISTENTLY OR CHRONICALLY INFECTABLE CELLS

An important criteria in selecting potential parent cell lines for the creation of alphavirus packaging cell lines, is the choice of cell lines that exhibit little or no cytopathological effects, prior to the appropriate production of alphavirus vector particles. This criteria is essential for the development of an alphavirus vector producer cell line which can be propagated for long periods of time and used as a stable source of vector. It is known that alphavirus infection of most mammalian cells results in cytopathology and lysis of the cell. However, the derivation of packaging cells from various insect cell lines may circumvent this problem. For example, insect cell lines, such as *Aedes*

*albopictus*, *Aedes aegypti*, *Spodoptera frugiperda*, and *Drosophila melanogaster* cells, may be utilized to construct packaging cell lines. For example, within one embodiment, alphavirus packaging cell lines are provided using an configuration uses an insect parent cell line, such as the *Aedes albopictus*, containing a stably transfected expression cassette vector which allows for expression of alphavirus structural proteins under the control of inducible or non-inducible promoters active in these cell types, and co-expressing a selectable marker.

Recently, a Sindbis virus-induced protein of cellular origin, which has been associated with the down-regulation of Sindbis virus production in some infected *Aedes albopictus* cells, has been identified and purified (Luo and Brown, *Virology* 194(1):44-49, 1993). The protein is a small hydrophobic peptide of approximately 3200 Da., which can induce an antiviral state and inhibit both 49S and 26S viral RNA synthesis. Cells treated with the antiviral peptide usually demonstrate quiescent arrest of cellular division for 96 hours in uninfected cells, and then normal growth rates are restored. Cells that have been exposed to this peptide prior to infection are unable to replicate Sindbis virus and appear to maintain this phenotype by constitutively producing the antiviral protein through 10 months of continuous passage.

It is recognized that this cellular response to Sindbis replication in *Aedes albopictus* cells might decrease the efficiency of a recombinant alphavirus vector producing system in those cells. To improve the efficiency of alphavirus vector production, two methods have been devised to inactivate the virus-induced cellular antiviral protein, thus preventing any reduction of vector particle titers. The first method entails purification of this cellular protein described above, and determination of a portion of the primary amino acid sequence using established techniques known in the art. The resulting amino acid sequence is then used to derive possible corresponding genomic sequences, enabling one to design a degenerate PCR primer pair which can be used to amplify the specific cellular sequence. This amplified sequence is then cloned using standard techniques known in the art, to obtain a discreet region of the gene encoding this inhibitory protein. Determination of the nucleotide sequence of this clone then enables one to design a vector which will integrate specifically within this Sindbis inhibitory gene by homologous recombination, and "knock out" its capacity to express a functional protein. Cell clones which contain the knock out sequence are identified by insertion of a selectable marker into the discreet cloned region of the inhibitory protein, prior to transfecting cells with the vector.

A second method for disabling this Sindbis virus inhibitory protein involves the treatment of *Aedes albopictus*-derived packaging cells with a mutagen, for example, BUDR (5-bromodeoxyuridine). The mutagenized packaging cell line population is then transfected or transduced with a Sindbis vector, which is able to express the neomycin resistance marker. Under high concentrations of the G418 drug, only those cells producing large amounts of Sindbis vector, and thus unable to express the Sindbis inhibitory gene, will be able to survive. After selection, resistant colonies are pooled, dilution cloned, and tested for high titer Sindbis production.

##### 2. MODIFICATION OF CELLS TO DECREASE SUSCEPTIBILITY TO ALPHAVIRUS EXPRESSION: SUPPRESSION OF APOPTOSIS AND CYTOPATHOLOGY

Packaging cell lines may also be modified by overexpressing the bcl-2 gene product in potential parent cell lines, such as canine D-17 and Cf2; human HT1080 and 293; quail

QT-6; baby hamster kidney BHK-21; mouse neuroblastoma N18; and rat prostatic adenocarcinoma AT-3. The conversion of these cells to a persistently infectable state allows for their use as alphavirus packaging and producer cell lines, similar to those of retrovector producer lines.

In order to construct such packaging cells, a bcl-2 expression vector is constructed by using standard recombinant DNA techniques in order to insert the 910 base pair Eco RI cDNA fragment derived from the plasmid p84 (*Nature* 336:259) into any commercially available expression vector containing a constitutive promoter and encoding a selectable marker, for example, pCDNA3 (Invitrogen, San Diego, Calif.). Careful consideration must be taken to avoid any type of homology between alphavirus nucleic acid sequences and other transduced vectors. This precaution should be taken in order to prevent recombination events which may lead to undesirable packaging of selectable markers or the bcl-2 oncogene in recombinant Sindbis particles. This is an important point, since the alphavirus vector system described herein is designed for use as a biological therapeutic. Once the bcl-2 expression vector is constructed, the parent cell line (i.e., BHK-21 cells) is transfected using any standard technique and selected after 24 hours using the appropriate marker. Resistant colonies are pooled, followed by dilution cloning, and then individual clones are propagated and screened for bcl-2 expression. Once expression is verified, persistent Sindbis infection is tested, followed by its use as a parent cell line for alphavirus packaging cell line development.

Other gene products, in addition to the bcl-2 oncogene, which suppress apoptosis may likewise be expressed in an alphavirus packaging or producer cell line. Three viral genes which are particularly preferred include: the adenovirus E1B gene encoding the 19-kD protein (Rao et al., *PNAS* 89:7742-7746, 1992), the herpes simplex virus type 1  $\gamma$ 34.5 gene (Chou and Roizman, *PNAS* 89:3266-3270, 1992), and the AcMNPV baculovirus p35 gene (Clem et al., *Science* 254:1388-1390, 1991). These individual genes may be inserted into any commercially available plasmid expression vectors, under the control of appropriate constitutive eukaryotic transcriptional promoters, and also containing a selectable marker, using standard techniques. The expression vector constructs are subsequently transfected into cell lines as described above, and the appropriate selection is applied. Selection for stable integration of these genes and constitutive expression their products should allow for more extended vector production in cell lines found to be susceptible to alphavirus-induced apoptotic events. In addition, it is feasible that each gene product inhibits apoptosis by its own unique mechanism. Therefore, the genes may also be introduced into packaging or producer cell lines in various combinations in order to obtain a stronger suppressive effect. Finally, other gene products having similar effects on apoptosis can also be readily incorporated into packaging cell lines as they are identified.

In the derivation of alphavirus vector packaging and producer cell lines, many approaches are outlined to control the expression of viral genes, such that producer cell lines stably transformed with both vector and vector packaging cassettes, can be derived. These approaches include inducible and/or cellular differentiation sensitive promoters, anti-sense structural genes, heterologous control systems, and mosquito or other cells in which viral persistent infections are established. Regardless of the final configuration for the alphavirus vector producer cell line, the ability to establish persistent infection, or at least delay cell death as a result of viral gene expression, may be enhanced by inhibiting apo-

ptosis. For example, the DNA tumor viruses, including adenovirus, HPV, SV40, and mouse polyomavirus (Py), transform cells in part, by binding to, and inactivating, the retinoblastoma (Rb) gene product p105 and its closely related gene product, p107, and other gene products involved in the control of the cell cycle including cyclin A, p33<sup>cdk2</sup> and p34<sup>cdc2</sup>. All of these viruses, except for Py, encode gene products which bind to and inactivate p53. Uniquely, Py encodes middle T antigen (mT) which binds to and activates the membrane tyrosine kinase, src, and also phosphatidylinositol-3-kinase, which is required for the full transformation potential of this virus (Talmage et al., *Cell* 59:55-65, 1989). The binding to and inactivation of the Rb and p53 recessive oncogene products prevents cells transformed by these DNA tumor viruses from entering the apoptotic pathway. It is known that p53 is able to halt the division of cells, in part by inhibiting the expression of proteins associated with cellular proliferation, including c-fos, hsc70, and bcl-2 (Miyashita et al., *Cancer Research* 54:3131-3135, 1994).

In order to extend the duration of alphavirus vector production, or to promote a persistently infectable state, packaging and producer cells are transformed with viral genomic DNA from Py or SV40. In particular, SV40 and Py transformed cell lines are established, and the kinetics and level of Sindbis production and cytopathology after viral infection determined. If apoptotic events characteristic of Sindbis proliferation in hamster cells are diminished, each prototype alphavirus packaging and producer cell line subsequently is transformed with Py or SV40, in order to increase the yield of packaged vector from these cells.

### 3. MODIFICATION OF CELLS TO DECREASE SUSCEPTIBILITY TO ALPHAVIRUS EXPRESSION: PRODUCTION OF ACTIVATION-DEPENDENT VECTOR PARTICLES

The Sindbis E2 glycoprotein is synthesized as a precursor, PE2. This PE2 precursor along with the second viral glycoprotein, E1, associate in the endoplasmic reticulum and are processed and transported to the infected cell membrane as a heterodimer for virion incorporation. At some point during this processing, PE2 is cleaved into E3 and the mature virion glycoprotein E2. E3 is the 64 amino-terminal residues of PE2 and is lost in the extracellular void during maturation. The larger cleavage product, E2, is associated with E1 and anchored in what becomes the viral envelope. Host cell protease(s) is responsible for processing of the PE2 precursor, cleaving at a site that immediately follows a highly conserved canonical four amino acid (aa) residue motif, basic-X-basic-basic aa's. A mutant cell line derived from the CHO-K1 strain, designated RPE.40 (Watson et al., *J. Virol* 65:2332-2339, 1991), is defective in the production of Sindbis virus strain AR339, through its inability to process the PE2 precursor into the E3 and mature E2 forms. The envelopes of Sindbis virions produced in the RPE.40 cell line therefore contain a PE2/E1 heterodimer. RPE.40 cells are at least 100-fold more resistant to Sindbis virus infection than the parental CHO-K1 cells, suggesting an inefficiency in the ability of PE2 containing virions to infect these cells. The defective virions produced by the RPE.40 cell line can be converted into a fully infectious form by treatment with trypsin.

In packaging and producer cell lines, any wild-type alphavirus that is produced by recombination between vector and structural protein gene RNAs will re-infect cells and be rapidly amplified; thus, significantly contaminating and decreasing the titer of packaged vector preparations. Packaging and producer cells developed from the RPE.40 line are

an alternative to other cell lines permissive for alphavirus infection due to the inefficient amplification of any wild-type virus generated during vector production and packaging. Thus, vector preparations are not significantly contaminated with wild-type virus. Furthermore, the benefits of this system are extended to other packaging and producer cell lines by developing "knock-out" mutants in their analogous cellular protease(s), using techniques known in the art.

#### 4. HOPPING CELL LINE DEVELOPMENT

Alphavirus hopping cell lines, as discussed previously, are used transiently to produce infectious RNA vector particles which have been pseudotyped for a different cellular receptor tropism. Once the hopping cell line produces vector particles, it is no longer required because only the infectious culture supernatants are needed to transduce the original alphavirus packaging cell lines discussed above. Therefore, the hopping cell line need not exhibit persistent infection by alphavirus in order to transiently produce vector particles. In this instance, the parent cell line can be either an insect cell line that exhibits persistent infection, or a mammalian cell line which is likely to lyse within 24–72 hours after a productive alphavirus infection. The only criteria is that the cell lines are able to express either VSV-G protein, with or without the appropriate alphavirus structural proteins, or retroviral gag-pol and env protein without affecting cell growth prior to introduction of the alphavirus RNA vector. Therefore, the alphavirus hopping cell line can be any of the aforementioned parent cell lines able to support either alphavirus or retroviral replication, without the additional cell modifications discussed previously, such as bcl-2 oncogene expression.

The generation of VSV-G pseudotyped alphavirus vector particles can be accomplished by at least three alternative approaches, two of which are dependent on the stable integration of a VSV-G expression cassette into cells. VSV-G protein is known to be highly cytotoxic when expressed in cells. Therefore, synthesis of this protein by the expression cassette is controlled by an inducible promoter. Specifically, a DNA fragment containing the VSV-G protein gene is isolated from plasmid pLGRNL (Emi et al., *J. Virol.* 65:1202–1207, 1991) by digestion with Bam HI, the termini made blunt using Klenow fragment enzyme and dNTPs, and the 1.7 kb fragment purified from a 1% agarose gel. Plasmid vector pVGELVIS-SINBV-linker (from Example 3), is digested with the enzyme Bsp EI to remove Sindbis non-structural protein coding sequences nts. 422–7054, and the remaining vector is re-ligated to itself to generate plasmid pVGELVISd1NSP-BV-linker. This plasmid is then digested with Xho I and the termini made blunt using Klenow fragment enzyme and dNTPs. The previously purified VSV-G fragment is subsequently ligated with this vector DNA, and resulting clones are screened for proper VSV-G insert orientation. This pVGELVIS-based VSV-G expression construct, in which VSV-G synthesis is controlled by a Sindbis replicon-inducible junction region, is designated pVGELVISd1-G.

Alternatively, a similar Sindbis replicon-inducible VSV-G expression cassette may be generated in the antisense configuration. In particular, plasmid vector pKSSINBV-linker (described in Example 3) is digested with the enzymes Apa I and Bam HI to most of the Sindbis nonstructural protein coding region, and the resulting 3309 bp vector fragment is purified from a 1% agarose gel. In addition, plasmid pd5'-26s (described in section B.3., this example) also is digested with the enzymes Apa I and Bam HI. The resulting 400 bp fragment which contains the HDV ribozyme/Sindbis 5' end fusion is purified from a 1% agarose gel and subsequently

ligated with the purified pKSSINBV-linker vector fragment to generate a plasmid designated pd5'-BVlinker. Plasmid pd5'-BVlinker is subsequently digested with Xho I, the termini made blunt using Klenow fragment enzyme and dNTPs, and ligated with the previously purified VSV-G fragment. The resulting construct, containing the expression cassette elements HDV antigenomic ribozyme/Sindbis 5'-end 299 nts./Sindbis junction region/VSV-G protein gene/Sindbis 3'-end untranslated region, is designated as plasmid pd5'-BV-G. Insertion of this VSV-G gene cassette into the pcDNA3 vector is as follows. Plasmid pd5'-BV-G is digested with the enzymes Pme I and Apa I, and the termini are made blunt by the addition of T4 DNA polymerase and dNTPs. The entire 2.5 kb VSV-G protein gene cassette is purified in a 1% agarose gel. Plasmid pcDNA3 is digested with the enzymes HindIII and Apa I and the termini are made blunt by the addition of T4 DNA polymerase and dNTPs, and the 5342 bp vector is purified in a 1% agarose gel. The two purified, blunt-end DNA fragments are subsequently ligated, and the resulting VSV-G protein gene expression cassette vector is known as plasmid pCMV/d5'VSV-G. Further modifications of the VSV-G expression cassettes pVGELVISd1-G and pCMV/d5'VSV-G to substitute other selectable markers, for example hygromycin resistance or *E. coli* gpt, for the current neomycin resistance, or other promoter elements, for example *Drosophila* metallothionein or hsp 70, for the current CMV, MuLV, and SV40 promoters, may be readily accomplished given the disclosure provided herein.

In a first VSV-G/alphavirus hopping cell line configuration, VSV-G expression cassette plasmid DNA (pVGELVISd1-G or pCMV/d5'VSV-G, or modified versions thereof) is transfected into the appropriate cell type (for example, BHK-21 cells) and selection for G418 resistance is applied using media containing 400 g/ml of G418 as described elsewhere in this example. G418-resistant cells are cloned by limiting dilution and the individual cell lines expanded for screening. VSV-G expressing cell lines are detected by transfection with any nonstructural protein gene-containing RNA vector (see Example 3) to induce the VSV-G expression cassette, followed by immunofluorescence using polyclonal rabbit anti-VSV antibody as described (Rose and Bergmann, *Cell* 34:513–524, 1983). The stably transfected VSV-G expressing cell line, in some cases, is subsequently transfected with plasmid expression cassette(s) which express one or more Sindbis structural proteins (described elsewhere in this example). For the production of VSV-G pseudotyped alphavirus particles, the appropriate vector RNA is transfected into the VSV-G hopping cell line, and vector particle-containing supernatants are recovered at least 24 hours post-transfection.

In a second VSV-G/alphavirus hopping cell line configuration, VSV-G expression cassette DNA (pVGELVISd1-G or pCMV/d5'VSV-G, or modified versions thereof) is transfected into previously derived alphavirus packaging cell lines (described elsewhere in this example) and the appropriate selection is applied as described previously. The selected cells are cloned by limiting dilution and the individual cell lines expanded for screening. VSV-G expressing cell lines are detected by transfection with any nonstructural protein gene-containing RNA vector (see Example 3) to induce the VSV-G expression cassette, followed by immunofluorescence using polyclonal rabbit anti-VSV antibody as described (Rose and Bergmann, *Cell* 34:513–524, 1983). For the production of VSV-G pseudotyped alphavirus particles, the appropriate vector RNA is transfected into the VSV-G hopping cell line,

and vector particle-containing supernatants are recovered at least 24 hours post-transfection.

In a third VSV-G/alphavirus hopping cell line configuration, VSV-G expression cassette DNA is co-transfected with the appropriate vector RNA into previously derived alphavirus packaging cell lines (described elsewhere in this example). Supernatants containing pseudotyped vector particles are recovered at least 24 hours post-transfection.

For the pseudotyping of alphavirus vectors in retroviral packaging cell lines, any cell line referenced in the literature, which expresses retroviral gag-pol and env sequences, may be used to package alphavirus RNA vector that has been engineered to contain a retroviral packaging sequence. The retrovirus psi packaging sequence is inserted between the inactivated junction region and a synthetic junction region tandem repeat, such that only genomic-length vector, and not subgenomic RNA, is packaged by the retroviral envelope proteins. Retroviral-based particles containing alphavirus vector RNA are produced by transfecting in vitro transcribed alphavirus vector RNA using procedures that have been described previously. Supernatants with pseudotyped retroviral particles containing alphavirus RNA vector are harvested at 24 hours post-transfection, and these supernatants are then used to transduce an alphavirus packaging cell line.

#### 5. IDENTIFICATION OF PARENT CELL LINES WHICH PRODUCE ALPHAVIRUS RESISTANT TO INACTIVATION BY HUMAN COMPLEMENT

Successful intravenous administration of recombinant alphavirus particles requires that the vector is resistant to inactivation in serum. It is well known to those skilled in the art that Sindbis grown on BHK cells is sensitive to inactivation, in terms of effective virus titer. In order to identify parent cell lines which produce Sindbis particles which are resistant to inactivation by human complement, the level of serum inactivation of Sindbis virus grown on multiple cell types is tested. The cell types tested are derived from many species, including human, for example, 293 or HT1080 (ATCC No. CCL 121).

As a source of human complement, approximately 70 mls of blood are collected from patients into serum separating tubes (Becton Dickinson, Los Angeles, Calif.). The blood is allowed to clot for one half hour at room temperature. After clotting the serum is centrifuged at 2000 g for 10 minutes at 4° C. The serum is collected and placed into a 15 ml conical tube (Coming, Corning, N.Y.) and placed on ice. Approximately, 1.1 ml aliquots of the serum are placed in 2 ml cryovials, frozen in a dry ice/ethanol bath and stored at -70° C. for subsequent serum inactivation assays. Complement inactivated controls are prepared by heat inactivation of control aliquots for 30 minutes at 56° C.

To test Sindbis for serum inactivation, two vials containing 1.1 ml of 100% non-heat inactivated human serum are used for various virus preparations. One vial of serum is quick thawed at 37° C. The serum is then heated to 56° C. for 30 minutes to heat inactivate complement present in the serum. Following inactivation the serum is placed on ice. The second vial is quick thawed at 37° C. After thawing the serum is placed on ice.

Approximately, 1.0 ml of the non-heat inactivated serum, medium, and heat-inactivated serum are placed in separate

1.5 ml tubes (Fisher Scientific, Pittsburgh, Pa.) and mixed with 10<sup>5</sup> Plaque Forming units (PFU) of Sindbis virus and incubated at 37° C. for 1 hour. After incubation the tubes are placed on ice.

In order to identify the parent cell line host from which an alphavirus is resistant to human serum inactivation, the non-heat inactivated serum, medium, and heat-inactivated serum virus preparations are titered by plaque assay on BHK cells. Equivalent virus titers regardless of incubation with non-heat inactivated serum, medium, or heat-inactivated serum, are indicative of parent cell line hosts from which Sindbis virus is resistant to human complement inactivation.

#### B. STRUCTURAL PROTEIN EXPRESSION CONSTRUCTS

##### 1. INDUCIBLE AND CONSTITUTIVE STRUCTURAL PROTEIN VECTOR EXPRESSION CASSETTES

The development of alphavirus packaging cell lines is dependent on the ability to synthesize high intracellular levels of the necessary structural proteins: capsid, pE2 and/or E2, and E1. Unfortunately, high level expression of these proteins, in particular, the envelope glycoproteins E2 and E1, may lead to concomitant cytopathology and eventual cell death. Therefore structural protein expression cassettes have been designed with inducible regulatory elements which control the levels of gene expression, in addition to others which maintain constitutive levels of expression.

In a first configuration, expression of the alphavirus structural proteins is under control of the RSV LTR, in conjunction with the inducible lac operon sequences. This is achieved by insertion of alphavirus cDNA corresponding to the viral structural protein genes into the pOP13 and pOPRSV1 vectors (Stratagene). These vectors, used separately, are co-transfected with the p3'SS vector (Stratagene), which expresses the lac repressor "i" protein. In the absence of inducer, for example, Isopropyl-B-D-thiogalactopyranoside (IPTG), the basal, or constitutive, level of expression of a luciferase reporter gene has been reported to be 10–20 copies per cell. Addition of IPTG, results in a conformational change of the repressor protein, which results in decreased affinity of the lac i protein for lac-operator sequences, permitting high level expression of the heterologous gene. Induction levels in the presence of IPTG of 95-fold have been reported for heterologous genes contained in the pOP13 vector.

Specifically, the Sindbis structural protein gene (SP) cDNA is inserted into the pOP13 and pOPRSV1 vectors as follows. The SP coding region is amplified in toto with a primer pair whose 5' ends map, respectively, to the authentic AUG translational start and UGA translational stop sites, including the surrounding nucleotides corresponding to the Kozak consensus sequence for efficient translational initiation at Sindbis nt 7638. The forward primer is complementary to Sindbis nts 7638–7661, and the reverse primer is complementary to Sindbis nts 11,384–11,364. PCR amplification of Sindbis cDNA corresponding to the structural protein genes is accomplished by a standard three-temperature cycling protocol, using the following oligonucleotide pair:

Forward primer (7638F):

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5'-TATATGGGGCCGACCAACCAACCATGAATAGAGGATTCTTTAATATGC-3'  
(SEQ. ID NO. 74)

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Reverse primer (11384R):

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5'-TATATGCGGCCGCTCATCTTCGTGTGCTAGTCAG-3'  
(SEQ. ID NO. 75)

---

In addition to their respective complementarities to the indicated Sindbis nts, a 5 nucleotide "buffer sequence" followed by the Not I recognition sequence is attached to the 5' ends of each primer. Following PCR amplification, the 3,763 bp fragment is purified in a 1% agarose gel, then subsequently digested with the Not I enzyme. The resulting 3,749 bp fragment is then ligated, separately, into the pOP13 and pOPRSV1 vectors, which are digested with Not I and treated with calf intestine alkaline phosphatase. These expression cassette vectors, which contain the entire coding capacity of the Sindbis structural proteins are known as pOP13-SINSP and pOPRSV1-SINSP.

Variations of the lac operon-Sindbis structural protein gene expression cassettes also can be constructed using other viral, cellular or insect-based promoters. Using common molecular biology techniques known in the art, the lac operon and the RSV LTR promoter, or just the RSV LTR promoter, sequences can be switched out of the Stratagene pOP13 and pOPRSV1 vectors and replaced by other promoter sequences, such as the cytomegalovirus major immediate promoter (pOPCMV-SINSP); the adenovirus major late promoter (pOPAMP-SINSP); the SV40 promoter (pOPSV-SINSP); or insect promoter sequences, which include the *Drosophila* metallothionein inducible promoter (pMET-SINSP), *Drosophila* actin 5C distal promoter (pOPA5C-SINSP), heat shock promoters HSP65 or HSP70 (pHSP-SINSP), or the baculovirus polyhedrin promoter (pPHED-SINSP).

## 2. MODIFICATION OF CASSETTES TO INCREASE PROTEIN EXPRESSION LEVELS

Alphavirus structural protein expression can be increased if the level of mRNA transcripts is increased. Increasing the level of mRNA transcripts can be accomplished by modifying the expression cassette such that alphavirus nonstructural proteins recognize these transcripts, and in turn, replicate the message to higher levels. This modification is performed by adding the wild-type minimal junction region core (nucleotides 7579 to 7602) to the extreme 5'-end of the Sindbis structural protein coding region, prior to the first authentic ATG start site for translation and inverting the expression cassette in the vector, so as to produce antisense structural protein gene transcripts. This can be accomplished by following the same PCR amplification technique described above for placing the Sindbis structural protein cDNA into the pOP13 and pOPRSV1 expression vectors. The only modification to this procedure is the replacement of the 7638F forward primer with a similar primer that includes junction region core nucleotides 7579-7602 between the Not I restriction enzyme site and the first ATG of the coding region as follows:

Forward primer (JUN7638F):

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5'-TATATGCGGCCGCTCATCTCTACGGTGGTCTAAATAGTACCACCACCATGAATAGAGGATTC-3'  
(SEQ. ID NO. 76)

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Following PCR amplification, the resulting 3,787 bp fragment is purified in a 1% agarose gel, then subsequently digested with the Not I enzyme. The resulting 3,773 bp fragment is then ligated, separately, into the pOP13 and

pOPRSV1 vectors which are digested with Not I and treated with calf intestine alkaline phosphatase. The resulting expression cassette vectors are known as pOP13-JUNSP and pOPRSV1-JUNSP. However, it must be stated that the introduction of junction region sequences into the structural protein expression cassettes will introduce sequences which may possibly lead to undesirable recombination events, leading to the generation of wild-type virus.

## 3. INDUCIBLE EXPRESSION OF STRUCTURAL PROTEINS VIA ALPHAVIRUS VECTOR

Because of potential cytotoxic effects from structural protein expression, the establishment of inducible packaging cell lines which express even modest basal levels of these proteins may not always be preferred. Therefore, packaging cell line expression cassettes are constructed which contain regulatory elements for the high level induction of structural protein synthesis via nonstructural proteins supplied in trans by the alphavirus vector, but with no basal level of synthesis until appropriately stimulated.

In this configuration, a structural protein gene cassette is constructed, whereby transcription of the structural protein genes occurs from an adjacent alphavirus junction region sequence. The primary features of this cassette are: an RNA polymerase II promoter positioned immediately adjacent to alphavirus nucleotide 1, such that transcription initiation begins with authentic alphavirus nucleotide 1, the 5'-end alphavirus sequences required for transcriptase recognition, the alphavirus junction region sequence for expression of the structural protein gene mRNA, the alphavirus structural protein gene sequences, the 3'-end alphavirus sequences required for replication, and a transcription termination/polyadenylation sequence. Because of an upstream open-reading frame which ends in translation termination codons prior to the AUG start site of the structural protein genes, expression of the alphavirus structural proteins can occur only after the synthesis of minus-strand RNA by vector-supplied nonstructural proteins, followed by the subsequent transcription of a structural protein gene mRNA from the junction region. Therefore, the inducibility of this system is dependent entirely on the presence of nonstructural proteins, supplied by the alphavirus vector itself, introduced as either RNA transcribed in vitro, or cDNA positioned downstream of an appropriate promoter element. In addition, the 5'- and 3'-end alphavirus sequences allow for this RNA transcript of the structural protein gene cassette to be amplified by the same vector-supplied nonstructural proteins (see FIG. 11).

Specifically, the construction of a positive-sense, vector-inducible Sindbis packaging cassette is accomplished as follows. Briefly, the pVGELVIS vector described previously is digested with the enzyme Bsp EI to remove nucleotides 422 to 7054, including most of the nonstructural gene coding sequences, and the remaining 9925 bp fragment is purified in a 0.8% agarose gel, and subsequently re-ligated to itself to generate the construct known as pLTR/SindBspE (FIG. 11). This deletion leaves the 5'-end authentic translation start codon at nts 60-62 intact, and creates in-frame downstream UAA and UGA stop codons at nts 7130-7132 and

7190-7192 (original numbering), respectively, thus preventing translation of the downstream structural protein gene open-reading frame. The pLTR/SindBspE packaging cassette construct is subsequently transfected into BHK cells



(ATCC #CCL 10) and transfectants are selected using the G418 drug at 400 ug/ml and cloned by limiting dilution. After expansion of the transfected clonal lines, screening for packaging activity is performed by transfection of Sindbis-luciferase (Sin-luc) vector RNA as described previously. The data shown in FIG. 12 demonstrate that transfection of Sin-luc vector RNA into several of these clonal LTR/SindBspE packaging cells results in the production of infectious Sindbis particles containing the Sin-luc RNA, as the recovered supernatants are shown to transfer Sin-luc vector RNA to fresh monolayers of BHK cells.

A similar packaging construct can also be made using the pVG-ELVISd clone (described previously) as initial material for creation of the Bsp EI deletion. In this clone, the Sindbis 3'-end sequence is followed by a catalytic ribozyme sequence to allow more precise processing of the primary transcript adjacent to the 3'-end sequences of Sindbis. In addition, a wide variety of variations of these packaging cassette constructions can be made given the disclosure provided herein, including for example, the substitution of other RNA polymerase promoters for the current MuLV LTR, the addition of 1 or more nucleotides between the RNA polymerase promoter and the first Sindbis nucleotide, the substitution of other ribozyme processing sequences, or the substitution of a non-Sindbis-encoded open reading frame upstream of the structural protein gene sequences, which may or may not retain the 5'-end Sindbis sequences required for transcriptase recognition. Furthermore, these constructs can be transfected into other cell lines, as discussed previously.

In another vector-inducible packaging configuration, expression cassettes contain a cDNA copy of the alphavirus structural protein gene sequences flanked by their natural junction and 3'-untranslated regions, and are inserted into an expression vector in an orientation, such that primary transcription from the promoter produces antisense structural protein gene RNA molecules. Additionally, these constructs contain, adjacent to the junction region, alphavirus 5'-end sequences necessary for recognition by the viral transcriptase, and a catalytic ribozyme sequence positioned immediately adjacent to alphavirus nucleotide 1 of the 5'-end sequence. As such, this ribozyme cleaves the primary RNA transcript precisely after the first alphavirus nucleotide. In this antisense orientation, the structural protein genes cannot be translated, and are dependent entirely on the presence of alphavirus virus nonstructural proteins for transcription into positive-strand MRNA, prior to their expression. These nonstructural proteins again are provided by the alphavirus vector itself. In addition, because this configuration contains the precise alphavirus genome 5'- and 3'-end sequences, the structural protein gene transcripts undergo amplification by utilizing the same nonstructural proteins provided by the alphavirus vector.

Specifically, the Sindbis structural protein gene cDNA is removed from the genomic clone pVGSP6GEN and inserted into the pcDNA3 (Invitrogen Corp., San Diego, Calif.) expression vector as follows. First, plasmid pVGSP6GEN is digested with the enzymes Apa I and Bam HI to remove all Sindbis sequences through nucleotide 7335, including the genes encoding nonstructural proteins 1, 2, 3, and most of 4. The remaining 7285 bp vector fragment, which contains the Sindbis structural protein genes, is purified in a 0.8% agarose gel, and subsequently ligated with a polylinker sequence, called SinMCS, that is obtained by annealing two synthetic oligonucleotides. The oligonucleotides, SinMCSI and SinMCSII, contain the recognition sites for Cla I, Bgl II, and Spe I, and have Apa I and Bam HI ends after annealing. Their sequences are as follows:

SinMCSI:

5'-CTCATCGATCAGATCTGACTAGTTG-3' (SEQ. ID NO. 77)

SinMCSII:

5'-CATCCAAGTACTAGTCAGATCTGATCGATGAGGGCC-3'  
(SEQ. ID NO. 78)

The resulting construct, known as pMCS-26s, is then modified to contain the 5'-end 299 nucleotides of Sindbis, fused to an 84 nucleotide ribozyme sequence from the antigenomic strand of hepatitis delta virus (HDV) (*Nature* 350:434), using overlapping PCR amplification. Two primer pairs are used initially in separate reactions, followed by their overlapping synthesis in a second round of PCR. In reaction #1, the forward primer (HDV49-XC) is complementary to HDV genome nucleotides 823-859, and the reverse primer (HDV17-68) is complementary to HDV genome nucleotides 839-887, with sequences as follows: Forward primer (HDV49-XC):

5'-ACTTATCGATGGTCTAGACTCCCTTAGCCATCCGAGTGGACG-  
TGCGTCCCTCCTC-3' (SEQ. ID NO. 79)

Reverse primer (HDV17-68):

5'-TCCACCTCCTCGCGTCCGACCTGGGCATCCGAAGGAGGACG-  
CAGCTCCACT-3' (SEQ. ID NO. 80)

In addition to their respective complementarities, primer HDV49-XC contains flanking Xba I and Cla I recognition sequences at the 5'-end. PCR amplification of HDV sequences is accomplished by a standard three-temperature cycling protocol with these primers and Vent polymerase. In reaction #2, the forward primer (SIN-HDV), which joins precisely the HDV and Sindbis sequences, is complementary to nucleotides 1-21 of Sindbis, and genomic nucleotides 871-903 of HDV, and overlaps the sequence of primer HDV17-68 (from above) by 20 nucleotides, and the reverse primer (SIN276-SPE) is complementary to Sindbis nucleotides 299-276, with sequences as follows: Forward primer (SIN-HDV):

5'-TCGGACCGCGAGGAGGTGGAGATGCCATGCCGAOCCATTGA-  
CGGCGTAGTACACT-3' (SEQ. ID NO. 81)

Reverse primer (SIN276-SPE):

5'-CTGGACTAGTTAATACTGGTGCTCGGAAAACATTCT-3'  
(SEQ. ID NO. 82)

In addition to their respective complementarities, primer SIN276-SPE contains a flanking UAA translation termination codon and Spe I recognition sequence at its 5' end. PCR amplification of the fragment containing Sindbis 5'-end sequences fused to HDV ribozyme sequences is accomplished by a standard three-temperature cycling protocol, using Vent polymerase, these primers, and pVGSP6GEN plasmid as template. After the first round of PCR amplification, 1/20th of the total amounts from each of reaction #1 and reaction #2 is combined and used as template in a second round of PCR amplification with additional



input of primers HDV49-XC and SIN276-SPE and a standard three-temperature cycling protocol. Following the second round of PCR, the 414 bp amplicon is purified with the MERMAID KIT (Bio101, La Jolla, Calif.), and digested with the enzymes ClaI and SpeI. The digested amplicon is purified in a 1% agarose gel, and subsequently ligated into plasmid pMCS-26s, which also is digested with ClaI and SpeI and purified in a 1% agarose gel. The resulting construct, containing the expression cassette elements HDV antigenomic ribozyme/Sindbis 5'-end 299 nts/Sindbis junction region/Sindbis structural protein genes/Sindbis 3'-end untranslated region, is known as pd5'26s.

Insertion of the structural protein gene cassette from pd5'26s into the pcDNA3 vector is performed as follows. Plasmid pd5'26s is digested with the enzyme Xba I and the 3'-recessed ends are made blunt by the addition of Klenow enzyme and dNTPs. The entire 4798 bp structural protein gene cassette is purified in a 1% agarose gel. Plasmid pcDNA3 is digested with the enzymes HindIII and Apa I and the ends are made blunt by the addition of T4 DNA polymerase enzyme and dNTPs, and the 5342 bp vector is purified in a 1% agarose gel. The two purified, blunt-end DNA fragments are subsequently ligated, and the resulting structural protein gene expression cassette vector is known as pCMV-d5'26s (see FIG. 11). Transfection of this DNA into cells and selection for G418 resistance is performed as previously described.

Modifications of the CMV promoter/antisense-Sindbis structural protein vector also can be constructed using other viral, cellular, or insect-based promoters. Using common molecular biology techniques known in the art, the CMV promoter can be switched out of the Invitrogen pcDNA3 vector and replaced by promoters such as those listed previously. Other variation of this antisense packaging cassette may include, but are not limited to: the addition of 1 or more nucleotides between the first Sindbis nucleotide and the catalytic ribozyme, the use of longer or shorter HDV or other catalytic ribozyme sequences for transcript processing, the substitution of a precise transcription termination signal for the catalytic ribozyme sequence, or the antisense expression of structural protein gene cassettes using any downstream sequence recognized by an RNA polymerase which results in transcription of a structural protein gene mRNA.

Further, it should be noted that each of the vector-inducible constructs described contains sequences homologous to the Sindbis vector itself. Therefore, the potential exists for the generation of wild-type virus by recombination between the two RNA molecules. Additional modifications may be made to eliminate this possibility as described below.

#### 4. SEPARATION OF STRUCTURAL PROTEIN GENES TO PREVENT RECOMBINATION

Packaging cell lines may also be generated which segregate the integration and expression of the structural protein genes, allowing for their transcription as non-overlapping, independent RNA molecules. For example, the expression of capsid protein independently of glycoproteins E2 and E1, or each of the three proteins independent of each other, eliminates the possibility of recombination with vector RNA and subsequent generation of contaminating wild-type virus.

Specifically, capsid protein is expressed independently from an inducible expression vector, such that sequences which might result in recombination with vector RNA are eliminated. As an example, the capsid protein gene is amplified from plasmid pVGSP6GEN with a primer pair complementary to nucleotides 7632-7655 (forward primer) and 8415-8439 (reverse primer), with sequences as follows:

Forward primer (Sin7632F):

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5'-GTCAAGCTTGCTAGCTACAACACCACCACCAATGAATAGAG-3'  
(SEQ. ID NO. 83)

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Reverse primer (Sin8439R):

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5'-CAGTCTCGAGTTACTACCACTCTTCTGTCCCTCCGGGGT-3'  
(SEQ. ID NO. 84)

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In addition to their respective complementarities, the forward primer contains Nhe I and HindIII recognition sequences at its 5'-end, and the reverse primer contains both UAG and UAA translation stop codons and a Xho I recognition sequence at its 5'-end. Amplification is accomplished using a standard three-temperature cycling protocol, and the resulting amplicon is digested with the enzymes Nhe I and Xho I, and purified in a 1% agarose gel. Expression plasmid pMAM (Clontech), which contains a dexamethasone-inducible MMTV LTR promoter sequence, is digested with the enzymes Nhe I and Xho I and the plasmid DNA purified in a 1% agarose gel. The capsid protein gene fragment is ligated into the pMAM vector, and the resulting construct is known as pMAM/C. Plasmid pMAM/C is transfected into the appropriate cell line (for example BHK-21) as described previously and selection for stable transfectants is accomplished by using HAT (hypoxanthine, aminopterin, thymidine) media, supplemented with dialyzed fetal calf serum, mycophenolic acid and xanthine, as described by Mulligan and Berg (PNAS 78:2072-2076, 1981). HAT-selected cell lines expressing capsid protein are identified following induction with  $10^{-6}$ M dexamethasone by lysing the cells with Lammeli sample buffer, separating the proteins using 12% SDS-PAGE, blotting onto nitrocellulose membrane, and detecting by western blot using polyclonal rabbit anti-Sindbis antibody. FIG. 21 shows expression of capsid protein in such cells, along with wild-type BHK-21 cells as a negative control, and Sindbis virus-infected BHK-21 cells as a positive control.

Alternatively, capsid protein is expressed using the lac-inducible vectors (Stratagene) described previously. The Sindbis capsid protein gene is amplified by PCR using primers Sin7632F and Sin8439R (described previously), and ligated with TA vector DNA (Stratagene). The resulting plasmid, designated TA/SinC, is digested with Eco RI, the termini are made blunt by the addition of Klenow fragment enzyme and dNTPs, and the capsid protein gene purified from a 1% agarose gel. Plasmid vectors pOP13 and pORSV1 are digested with Not I, their termini made blunt by the addition of Klenow fragment enzyme and dNTPs, and subsequently treated with calf intestinal alkaline phosphatase. The capsid protein gene is ligated with both pOP13 and pORSV1 vector DNA to generate the expression constructs designated pOP13CAP and pORSV1CAP, respectively. Each plasmid is co-transfected with p3'SS into the appropriate cell line as described previously, and selection for stable transfectants is accomplished using G418 and hygromycin selection. Cell lines expressing capsid protein are identified following IPTG induction by immunofluorescence using polyclonal rabbit anti-Sindbis antibody.

The glycoprotein genes, E1 and E2, are expressed together using one of the inducible systems previously described. For example, the Sindbis E1 and E2 genes are amplified from plasmid pVGSP6GEN using a primer pair complementary to Sindbis nucleotides 8440-8459 (forward primer) and Sindbis nts 11,384-11,364 (reverse primer).

PCR amplification is performed using a standard three-temperature cycling protocol and the following oligonucleotide pair:

Reverse primer (1 1384R):

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5'-TATATGCGGCCGCTCATCTCGTGTGCTAGTCAG-3'  
(SEQ. ID NO. 75)

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Forward primer (8440F):

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5'-TATATGCGGCCGCAACCATGTCGACAGCACTGGT-  
CAAG-3' (SEQ. ID NO. 85)

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In addition to their respective complementarities, the forward primer contains an "in-frame" AUG translation initiation codon, and both primers contain a NotI recognition sequence at their 5'-ends. Following PCR amplification, the amplicon is digested with the NotI enzyme and purified in a 1% agarose gel. The resulting fragment is then ligated separately into the pOP13 and pOPRSV1 vectors (Stratagene), digested with NotI and treated with calf intestinal alkaline phosphatase, as described previously. These glycoprotein expression vectors are used to transfect cells that have been previously transfected with a capsid protein expression construct, and stable glycoprotein gene transfectants are identified by selection for G418 and hygromycin resistance.

Alternatively, the E1 and E2 glycoproteins are expressed under the control of the replicon-inducible junction region promoter, described previously. The ELVIS expression plasmid pVGELVISOSINBV-linker (Example 3) is digested with the enzyme NotI, and treated with calf intestinal alkaline phosphatase. PCR amplified Sindbis E1 and E2 glycoprotein genes digested with NotI (previous paragraph) are then ligated to the ELVIS vector to generate a construct designated pVGELVIS-E1/E2. Plasmid pVGELVIS-E1/E2 subsequently is digested with the enzyme BspEI, removing most of the nonstructural protein gene coding region, and the remaining E1- and E2-containing vector DNA is re-ligated to itself, creating an inducible expression cassette identified as pVGELVd1-E1/E2. This glycoprotein expression vector is used to transfect cells that have been previously transfected with a capsid protein expression construct, and stable glycoprotein gene transfectants are identified by selection for G418 resistance. For both the capsid and envelope glycoprotein expression cassettes, additional mammalian or non-mammalian (including insect)-derived promoters, which may or may not be inducible, are readily substituted for those described above, using standard techniques known in the art.

#### 5. ASSEMBLING THE COMPONENTS TO CREATE THE ALPHAVIRUS PACKAGING CELL LINE

For example purposes, the BHK-21 cell line and replicon-inducible packaging expression cassette are used to demonstrate assembly of the components. However, other possible parent cell lines can be used to create alphavirus packaging cell lines and have been discussed previously. Briefly, BHK-21 cells (CCL 10) are grown at 37° C. in 5% CO<sub>2</sub> in Dulbecco's modified Eagle's Media (DMEM), 2 mM L-glutamine, and 10% fetal bovine serum (optimal media). Approximately 5x10<sup>5</sup> BHK-21 cells, grown in a 35 mm petri dish, are transfected with 5 ug pLTR/SindIBspE using 5 ul of the Transfectam (Promega) cationic lipid reagent, in serum-free media conditions, as suggested by the supplier. However any method of transfection is rapidly substituted, i.e., by electroporation, calcium phosphate precipitation, or

by using any of the readily available cationic liposome formulations and procedures commonly known in the art. At 24 hours post-transfection, the cells are trypsinized and reseeded in 100 mm dishes in 10 ml of optimal media, as described above, supplemented with 400 ug/ml of G418 (Gibco/BRL) and selected over a period of 5 to 7 days. Colonies displaying resistance to the G418 drug are then pooled, dilution cloned, and propagated. Individual clones are screened for high levels of Sindbis structural protein expression and functional packaging after transfection with Sindbis-luciferase vector RNA transcribed in vitro from SacI linearized plasmid pKSSINBV-luc (see Example 3). Specifically, clonally-derived pLTR/SindIBspE transfected BHK-21 cells (referred to as LTR/SindIBspE or BK-Bsp cells) grown in 60 mm petri dishes are transfected with 2 ug of Sindbis-luciferase vector RNA and overlaid with 3 ml of optimal media (see above). At 20 hours post-transfection, the supernatants are removed, and clarified by centrifugation for 30 min. at 3000 rpm in a Sorvall RT6000B tabletop centrifuge. In addition, the transfected cell monolayer is lysed in reporter lysis buffer (Promega) as described by the manufacturer, and assayed for luciferase expression as described previously.

The transfer of luciferase activity (and thus functional packaging) is tested by using 1 ml of the above supernatants to infect fresh monolayers of BHK-21 cells in 60 mm dishes. At 20 hours post-infection, the cell monolayers are lysed as described above, and tested for luciferase expression. As shown in FIG. 12, three clones (#13, 18, and 40) produce packaged Sindbis-luciferase vector and are the first examples of alphavirus packaging cell lines. In addition, transfected clone #18 cells are tested for increased vector packaging over a timecourse following transfection. Supernatants from transfected clone #18 cells are harvested at 20, 45, and 70 hours post-transfection, as described above, and used to infect fresh monolayers of BHK-21 cells. FIG. 13 shows that Sindbis-luciferase vector packaging increases significantly at 45 hours post-transfection, as compared to 20 hours post-transfection. Expression also can be tested by western blot analysis using polyclonal rabbit anti-Sindbis antibodies (available in the literature).

#### C. INDUCIBLE VECTOR AND STRUCTURAL PROTEIN EXPRESSION FOR ALPHAVIRUS PRODUCER CELL LINES

##### 1. USE OF VIRAL PROMOTERS

The challenge of developing an alphavirus vector producer cell line lies in the question of whether a virus, whose infection of mammalian cells results almost exclusively in productive lytic cell death, can be modified to establish persistent infection in these same cells. One approach is to generate alphavirus vector producer lines from mosquito cells, where viral persistence often results after infection. However, the titer of infectious virus produced in persistently infected mosquito cells is only about 1x10<sup>4</sup> PFU/ml, at least five orders of magnitude less than that observed after lytic infection of BHK cells by Sindbis.

Several strategies are described for inducible alphavirus vector producer cell lines, containing both vector and viral structural gene cassettes, such that productive cytolytic infection occurs only after the correct stimulus. Because these approaches operate on a "feed forward" level, any leakiness in the system will result in initiation of the alphavirus replication cycle and probable cell death. Therefore, tightly regulated control mechanisms are necessary for such a system.

The hallmark of development is the differentiation state-dependent pattern of gene expression. Briefly, gene expres-

sion patterns differ widely between undifferentiated and terminally differentiated states. Thus, a cell whose differentiation state can be controlled is likely an ideal host in which to derive an alphavirus vector producer cell line. In such a configuration, the vector expression cassette and, in some instances, structural components are coupled to terminal differentiation state-inducible promoters, according to the strategy described for ELVIS, and used to transform stably an undifferentiated host cell. Terminal differentiation of the host producer cell after induction with the appropriate stimuli coincidentally results in induction of the alphavirus replication cycle and production of packaged vector. Other strategies described herein, including antisense structural genes and heterologous viral expression systems, are readily coupled with cellular differentiation state-dependent promoters described below.

In this approach, four examples are described, using either a viral or cellular promoter which are active in only terminally differentiated cells.

It has been shown that mouse Polyomavirus (Py), SV40, and Moloney murine leukemia virus (M-MuLV), all are able to infect and enter undifferentiated mouse embryonal carcinoma (EC) cells, but the expression of their genes (and heterologous genes) and establishment of productive infection is blocked (Swartzendruber and Lehman, *J. Cell. Physiol.* 85:179-188, 1975; Peries et al., *J. Natl. Cancer Inst.* 59:463-465, 1977). These viral growth properties also have been demonstrated in two cell lines, PCC4 and F9, which are derived from the malignant stem cells of mouse teratocarcinomas. The block to viral propagation occurs at the level of transcription and replication, and maps to the enhancers, contained within the viral non-coding control regions (Linney et al., *Nature* 308:470-472, 1984; Fujimura et al., *Cell* 23:809-814, 1981; Katinka and Yaniv, *Cell* 20:393-399, 1980). When M-MuLV infects undifferentiated EC cells, the viral DNA integrates into the genome. However, as stated above, expression of viral genes or of heterologous genes is blocked. This block of viral expression is released upon terminal differentiation of EC cells by addition of retinoic acid to the growth medium.

To test the RNA expression properties of the pVGELVIS construct in EC cells, plasmid DNA is complexed with LIPOFECTAMINE (GIBCO-BRL, Gaithersburg, Md.) according to the conditions suggested by the supplier (ca. 5 g DNA/8 g lipid reagent) and added to 35 mm wells containing undifferentiated PCC4 or F9 cells (Fujimura et al., 1981, *Cell* 23:809-814) at approximately 75% confluency. The development of cytopathic effects (CPE), and the level of Sindbis productive infection, quantitated by plaque assay of media supernatant, is determined at regular intervals over 5 days in undifferentiated and differentiated transfected PCC4 or F9 cells. Differentiation of F9 and PCC4 cells is accomplished by addition of retinoic acid (Sigma Chemical Co., St. Louis, Mo.), at a final concentration of 1M.

It has been proposed that the hierarchy of relative expression of heterologous genes observed in undifferentiated EC cells infected with M-MuLV vectors may be in part insertional dependent (Linney et al., 1987, *J. Virol.* 61:3248-3253). Thus, undifferentiated EC cells transfected with pVGELVIS may likely produce different results, in terms of transcription of the Sindbis genomic cDNA and, in turn, initiation of the viral life cycle. In this event, following G418 selection of pVGELVIS transfected undifferentiated EC cells, remaining cells are cloned and expanded. The cell clones are then tested for the production of Sindbis virus after differentiation by addition of retinoic acid (Sigma Chemical Co., St. Louis, Mo.), at a final concentration of 1M.

To isolate vector packaging cell lines, whose production of structural proteins in the presence of Sindbis NSP is cell differentiation state dependent, undifferentiated F9 or PCC4 cells are transfected with pLTR/SINdIBspE and G418 selected as described above. Differentiation state-sensitive clones are then selected by infection at high multiplicity with packaged SIN-luc vector. Clones which are resistant to cell lysis or do not produce packaged SIN-luc vector particles, are candidate vector packaging clones. These candidate clones are tested for SIN-luc vector particle production following terminal differentiation with retinoic acid, as described.

The murine wild type polyomavirus (Py) is unable to replicate in the teratocarcinoma cell lines PCC4 or F9. This block of replication in undifferentiated cells occurs at the level of transcription of early region (i.e., T antigen) genes, and is released by induction of terminal differentiation with vitamin A. Py mutants which are able to establish productive infection in undifferentiated PCC4 and F9 cells map to the viral enhancer region. The genesis of an embryonic tissue specific transcriptional enhancer element has resulted in these mutants. In order to exploit this property of inhibition of Py replication in undifferentiated teratocarcinoma cell lines, the viral regulatory non-coding region, including the enhancer, is coupled to the genomic cDNA of Sindbis virus, according to the ELVIS strategy. The precise transcriptional start site of the Py early region has been determined (see Tooze, *DNA Tumor Viruses*). The PCC4 and F9 cell lines are stably transformed with the Py-Sindbis vectors. In this model Sindbis productive infection occurs after addition of retinoic acid to the culture medium and induction of terminal differentiation.

The Py non-coding region from bases 5021-152, which includes the sequences corresponding to the viral enhancers, 21 bp repeats, replication origin, CAAT and TATA boxes and the early mRNA transcription 5' cap site, is positioned at the 5' viral end such that *n vivo*, only a single capped C residue is added to the Sindbis 5' end. Juxtaposition of the Py non-coding region and the Sindbis 5' end is accomplished by overlapping PCR as described in the following detail. Amplification of the Py non-coding region in the first primary PCR reaction is accomplished in a reaction containing the pBR322/Py, strain A2 plasmid (ATCC number 45017-p53.A6.6(pPy-1)) and the following primer pair:

Forward primer: Pybg15021F (buffer sequence/Bgl II recognition sequence/Py nts 5021-5043):

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5'-TATATAGATCTCTTGATCAGCTTCAGAAGATGGC  
(SEQ. ID NO. 86)

---

Reverse primer: SINPy152R (SIN nts 5-1/Py nts 152-134):

---

5'-TCAATGGCGGAAGAGGCGGTTGG (SEQ. ID NO. 87)

---

PCR amplification of the Py non-coding region with the primer pair shown above is performed using the Thermalase thermostable DNA polymerase (Amersco Inc., Solon, Ohio) and the buffer containing 1.5 mM MgCl<sub>2</sub>, provided by the supplier. Additionally, the reaction contains 5% DMSO, and the Hot Start Wax beads (Perkin-Elmer), using the following PCR amplification protocol shown below:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	0.5	
72	10	1

Amplification of the Sindbis 5' end in the second primary PCR reaction is in a reaction containing the pVSP6GEN clone and the following primer pair:  
Forward primer: (Py nts 138-152/SIN nts 1-16):

5'-CCGCCTCTTCCGCCATTGACGGCGTAGTAC (SEQ. ID NO. 88)

Reverse primer: (SIN nts 3182-3160):

5'-CTGGCAACCGGTAAGTACGATAC (SEQ. ID NO. 18)

PCR amplification of Sindbis 5' end region with the primer pair shown above is with the reaction conditions described above, using the following PCR amplification below:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	3.0	
72	10	1

The 442 bp and 3202 bp products from the primary PCR reactions are purified with GENECLEAN (BIO 101), and used together in a PCR reaction with the following primer pair:

Forward primer: Pybgl5021F (buffer sequence/Bgl II recognition sequence/Py nts 5021-5043):

5'-TATATAGATCTCTTGATCAGCTTCAGAAGATGGC (SEQ. ID NO. 89)

Reverse primer: (SIN nts 2300-2278):

5'-GGTAACAAGATCTCGTGCCGTG (SEQ. ID NO. 19)

PCR amplification of the of the primer PCR amplicon products with the primer pair shown above is with the reaction conditions described above, using the following PCR amplification protocol shown below:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	3.0	
72	10	1

The 20 3' terminal bases of the first primary PCR amplicon product overlaps with the 20 5' terminal bases of the second primary PCR amplicon product; the resultant 2,742 bp overlapping secondary PCR amplicon product is purified

by 0.8% agarose/TBE electrophoresis, digested with Bgl II, and the 2,734 bp product is ligated into pcDNASINbgl/xba (see Example 3) treated with Bgl II and CIAP. The resulting construction is 16,641 bps and is known as ELVIS-PySIN. In order to construct a structural protein expression vector similar to pLTR/Sind1Bsp for the derivation of vector packaging cell lines, the ELVIS-PySIN construction is digested to completion with Bsp EI, and religated under dilute conditions, in order to accomplish deletion of the nonstructural proteins between bases 422-7054. This construction is known as ELVIS-PySINd1BspE.

ELVIS-PySIN plasmid DNA is complexed with LIPO-FECTAMINE (GIBCOBRL, Gaithersburg, Md.) according to the conditions suggested by the supplier (ca. 5 g DNA/8 g lipid reagent) and added to 35 mm wells containing undifferentiated PCC4 or F9 cells at approximately 75% confluency. The development of cytopathic effects (CPE), and the level of Sindbis productive infection, quantitated by plaque assay of media supernatant, is determined at regular intervals of 5 days in undifferentiated and differentiated PCC4 or F9 cells. Differentiation of F9 and PCC4 cells is accomplished by addition of retinoic acid (Sigma Chemical Co., St. Louis, Mo.), at a final concentration of 1 mM.

If the undifferentiated EC cells demonstrate a heterologous response to transfection with ELVIS-PySIN, remaining cells not lysed by Sindbis virus propagation following G418 selection of pVGELVIS transfected undifferentiated EC cells are cloned and expanded. The cell clones are then tested for the production of Sindbis virus after differentiation, by addition of retinoic acid (Sigma Chemical Co., St. Louis, Mo.), at a final concentration of 1 mM.

Isolation of vector packaging cell lines stably transfected with ELVIS-PySINd1BspE, having a cell differentiation state dependent pattern of expression of structural proteins in the presence of Sindbis NSP, is accomplished as described above for the pLTRISind1BspE plasmid.

In order to demonstrate the feasibility of an inducible Sindbis vector producer cell line, the reporter gene expression from the ELVIS-luc vector, whose construction is described in Example 3, section E, after transfection of BHK and undifferentiated F9 cells is determined. In addition, both cell types are infected with packaged SIN-luc vector, whose production is described in Example 3 section C. This later experimental group serves as a control that expression restriction (if any) lies at the level of transcription rather than a receptor difference on unique cell types. The results of this study, shown in FIG. 14, demonstrate that the expression of luciferase is inhibited in undifferentiated F9 cells. The level of luciferase expression in BHK cells transfected with ELVIS-luc and BHK and undifferentiated F9 cells infected with packaged SIN-luc vector is similar. Thus, in ELVIS-luc transfected undifferentiated F9 cells, transcription from the LTR and subsequent luciferase expression via the Sindbis vector autocatalytic pathway is inhibited. This study demonstrates that packaging cell lines can be developed where synthesis of Sindbis vector or Sindbis vector packaging is inducible and controlled by the differentiation state of the cell.

## 2. USE OF CELLULAR PROMOTERS.

The third example of this strategy uses the  $\beta$ -globin locus control region. The  $\beta$ -globin multigene cluster contains five developmentally regulated genes. In the early stages of human development, the embryonic yolk sac is the hematopoietic tissue and expresses the  $\epsilon$ -globin gene. This is followed by a switch to the  $\gamma$ -globin gene in the fetal liver and the  $\delta$ - and  $\beta$ -globin genes in adult bone marrow (Collins and Weissman, 1984, *Prog. Nucleic Acid Res. Mol. Biol.* 31:315).

At least two mouse erythroleukemia lines, MEL and Friend, serve as models for terminal differentiation dependent expression of  $\beta$ -globin. Expression of  $\beta$ -globin is observed in these lines only after induction of terminal differentiation by addition of 2% DMSO to the growth medium.

The entire  $\beta$ -globin locus is regulated by the locus control region (LCR). Within the LCR is the dominant control region (DCR) residing within the DNase I hypersensitive region, which is 5' of the coding region. The DCR contains five DNase I hypersensitive (HS1-HS5) sites. The DCR directs high level site of integration independent, copy number dependent expression on a linked human  $\beta$ -globin gene in transgenic mice and stably transfected mouse erythroleukemia (MEL) cells (Grosveld et al., 1993, *CSHSQB* 58:7-12). In a recent study (Ellis et al., 1993, *EMBO* 12:127-134), concatamers of a synthetic core coinciding to sequences within HS2 were shown to function as a locus control region.

In order to accomplish the differentiation state dependent expression of alphavirus vectors, the viral genomic cDNA is juxtaposed with a promoter containing a tandem synthetic core corresponding to the LCR HS2 site. Alternatively, the desired alphavirus vector construct can be inserted downstream of the LCR in the endogenous-globin gene by homologous recombination. In such a strategy, the  $\beta$ -globin transcription initiation site after terminal differentiation would be first determined, in order that the alphavirus vector could be placed precisely at the start site.

Initiation of a lytic viral life cycle is controlled by the differentiation state of the host cell is applicable to other systems, where the control of viral induced cytopathology is desired.

Yet another approach to regulating alphavirus gene expression through a differentiation state sensitive promoter is the use of the retinoic acid receptor a (RARA) and acute promyelomonocytic leukemia cells (APL). APL cells are clonal myeloid precursors characterized by high growth rate and differentiation arrest. A non-random chromosomal translocation breakpoint, t(15;17)(q22;21), occurs in almost all patients with APL. The RARA gene has been localized to chromosome 17q21. Analysis of APL mRNA from patients has shown that most APL breakpoints occur within the second intron of the RARA gene and result in abnormal fusion transcripts. Co-transfection assays with RARA and PML-RARA fusion cDNAs have demonstrated that the resulting fusion proteins can antagonize wild-type RARA in the presence of retinoic acid. These studies implicate PML-RARA fusion protein in the molecular pathogenesis of APL. Importantly, a significant number of patients achieve complete remission after all-trans retinoic acid treatment (ATRA). High concentration of ATRA may overcome the RARA deficiency leading to high levels of RA in the nucleus. Differentiation of the APL cells can then be achieved through activation of RARA responsive genes. RA can induce differentiation of a number of cell lines, including the human leukemia line HL-60.

The retinoic acid receptor is a member of a nuclear receptor superfamily that includes the thyroid and steroid hormone receptors. Four different forms of the human RAR have been identified, and the corresponding cDNAs cloned and characterized. In order to accomplish the differentiation state dependent expression of Sindbis vectors, viral genomic cDNA is juxtaposed with the RARA DNA binding site, creating ELVIS-RARASIN. As with the strategy proposed for ELVIS-PySIN expression in undifferentiated EC cells, differentiation sensitive ELVIS-RARASIN expressing cells are isolated.

### 3. INSERTION OF VECTOR CONSTRUCTS INTO DIFFERENTIATION STATE CONTROLLED INDUCIBLE PROMOTERS.

Generation of clones whose expression of heterologous genes from Sindbis vectors positioned in the ELVIS configuration as described in Example 3 is differentiation state dependent, is accomplished as described above for the pVGELVIS, pLTR/Sind1BspE plasmids. Generation of clones whose production of vector particles is differentiation state dependent, is accomplished by transfecting the isolated differentiation dependent vector packaging clones described above with ELVIS heterologous gene expression vectors. Clones having the desired phenotype or vector production after retinoic acid induced differentiation are isolated as described above.

### D. STRUCTURAL PROTEIN EXPRESSION FROM A HETEROLOGOUS ASTROVIRUS JUNCTION REGION.

Among the critical properties of a vector packaging system are a cell line which expresses the structural components necessary to generate an infectious particle, without the creation of wild-type virus through recombination between vector and structural gene components. These two desired properties of the packaging cell line are accomplished in the retrovirus based systems through the constitutive expression of the gag/pol and env genes on individual heterologous RNA polymerase II expression cassettes.

Another important aspect of vector packaging cell lines is to derive a system which mimics as closely as possible the normal replication strategy of the wild type virus. This issue is important in terms of the observed titer level of packaged recombinant vector. Synthesis of the viral structural proteins during alphavirus infection is accomplished after transcription of high levels of subgenomic mRNA from the junction region promoter, followed by efficient translation into the structural proteins. The junction region promoter is functional only in the antisense orientation and synthesis of the antigenomic RNA occurs after translation of the nonstructural proteins, thus delaying the expression of the structural proteins. It follows that, with regard to alphavirus, it would be desirable to construct a packaging cell line in which synthesis of the structural proteins is initiated from the junction region promoter, which in turn is activated by nonstructural proteins expressed from the recombinant vector molecule.

It is known that a relatively high frequency of recombination occurs between RNA genomic molecules occurs during infection with Sindbis virus via a copy choice mechanism (*PNAS* 88:3253-3257, 1991). Recombination between vector and junction region/structural gene cassettes would result in the generation of wild-type Sindbis virus, perhaps at a level of 1 wild-type virus per million of packaged vector particles (*Liljestrom Bio Technology* 9:1356-1361, 1991). One way to mitigate the generation of wild-type virus is to separate the structural genes onto separate expression cassettes, an approach which has been discussed previously in Example 7.

An additional approach to diminish the level of wild-type virus production in alphavirus vector packaging cell lines is to express the structural proteins under the control of Astrovirus genetic elements. A schematic for this configuration is depicted in FIG. 15. Similar to alphaviruses, the expression of Astrovirus structural proteins incorporates a junction region strategy, in which high levels of structural proteins are synthesized from a subgenomic message. The Astrovirus expression cassette may consist of one of the two following ordered elements: (1) inducible promoter/Astrovirus 5' end/Astrovirus junction region/alphavirus structural gene/

Astrovirus 3' end, or (2) antisense Astrovirus 3' end/antisense alphavirus structural gene/antisense Astrovirus junction region/antisense Astrovirus 5' end/ Hepatitis Delta virus ribozyme, or other configurations described in Example 7. In both configurations, the expression unit is amplified by the Astrovirus nonstructural proteins through the same mechanism that occurs during viral replication. Since multiple rounds of subgenomic mRNA synthesis initiated from the junction region occur from each expression unit, amplification of the expression unit by the Astrovirus nonstructural proteins results in the production of very high levels of alphavirus structural proteins. The second configuration of the alphavirus structural protein expression cassette described above may function better than the first, because the primary transcript of the toxic alphavirus structural gene is antisense. Although expression of the structural genes in the first configuration should not occur until synthesis of the negative strand followed by synthesis of the positive subgenomic RNA from the junction region, the antisense nature of the primary transcript in the second configuration represents an additional level of control to prevent cytotoxic protein expression.

It is likely that no wild-type virus would be generated in a packaging cell line in which the alphavirus virus structural proteins are synthesized individually from Astrovirus junction region expression cassettes. Recombination between the nonstructural protein region of the vector and an Astrovirus structural protein expression cassette would result in a molecule in which Astrovirus cis elements were coupled with alphavirus genes, a nonviable combination. Correct coupling of alphavirus cis and trans elements would require two precise recombination events between the vector and the Astrovirus expression cassette, between the Astrovirus junction region and structural gene ATG, and between the structural gene termination codon and the Astrovirus 3' end. In order to generate wild type virus, this dual recombination event would have to occur three times on the same molecule (six total events), to incorporate the three separated alphavirus structural genes.

In order to diminish any possible toxicity of the Astrovirus proteins, synthesis of the Astrovirus expression cassettes may also be controlled by inducible promoters. One possibility is to use the lac operon, according to the "lac-switch" system described previously in Example 7 (Stratagene). The constitutive level of expression of the lac operon controlled gene in the absence of the gratuitous inducer IPTG is about 10 copies of RNA per cell. The inducible promoter corresponding to the Astrovirus/alphavirus structural gene expression cassette may be the lac operon or other suitable promoters which have very low level of constitutive expression. Construction of packaging cell lines of these configurations, in which the control of alphavirus proteins is directed by a heterologous virus should result in the generation of high titer wild-type virus free packaged vector particles.

#### Example 8

##### ALTERNATIVE VIRAL VECTOR PACKAGING TECHNIQUES

Various alternative systems can be used to produce recombinant alphavirus particles carrying the vector construct. Each of these systems takes advantage of the fact that baculovirus, and the mammalian viruses vaccinia and adenovirus, among others, have been adapted recently to make large amounts of any given protein for which the gene has been cloned. (Smith et al., *Mol. Cell. Biol.* 3:12, 1983;

Piccini et al., *Meth. Enzymology* 153:545, 1987; and Mansour et al., *Proc. Natl. Acad. Sci. USA* 82:1359, 1985). These and other viral vectors are used to produce proteins in tissue culture cells by insertion of appropriate genes into the viral vector and can be readily adapted to make alphavirus vector particles.

For example, adenovirus vectors are derived from nuclear replicating viruses and can be modified so they are defective. Heterologous genes are inserted into these vectors either by in vitro construction (Ballay et al., *EMBO J.* 4:3861, 1985) or by recombination in cells (Thummel et al., *J. Mol. Appl. Genetics* 1:435, 1982), and used to express proteins in mammalian cells. One preferred method is to construct plasmids using the adenovirus major late promoter (MLP) driving: (1) alphavirus structural proteins; and (2) an alphavirus vector construct. The alphavirus vector in this configuration still contains a modified junction region, and would allow the transcribed RNA vector to be self-replicating, as in previously described configurations.

These plasmids are then used to make adenovirus genomes in vitro (Ballay et al., *EMBO J.* 4:3861, 1985). The recombinant adenoviral genomes, which are replication defective, are separately transfected into 293 cells (ATCC #CRL 1573, a human cell line making adenovirus E1A protein), to yield pure stocks of defective adenovirus vectors expressing either alphavirus structural proteins or alphavirus vectors. Since the titers of such vectors are typically  $10^7$ - $10^{11}$ /ml, these stocks are then used to infect tissue culture cells simultaneously at high multiplicity of infection, resulting in the production of alphavirus proteins and vector genomes at high levels. Since the adenovirus vectors are defective, little or no direct cell lysis will occur and vectors are harvested from the cell supernatants. Similar approaches are readily carried out using recombinant vaccinia virus vectors constructed by inserting the alphavirus sequences into the shuttle plasmid pK (Bergmann et al., *Eur. J. Immunol.* 23:2777, 1993) for in vivo recombination into the vaccinia WR strain.

Other viral vectors, such as those derived from unrelated vectors (e.g., RSV, MMTV or HIV), also may be used in the same manner to generate packaged vectors from primary cells. In one embodiment, these adenoviral vectors are used in conjunction with primary cells, giving rise to recombinant alphavirus particles.

An alternative expression system also has been described in which chimeric HIV/poliovirus genomes result in the generation of chimeric minireplicons (*J. Virol.* 65:2875, 1991) capable of expressing fusion proteins. These chimeric poliovirus minireplicons, in which HIV-1 gag-pol sequences were substituted for the VP2 and VP3 capsid genes of the P1 capsid of poliovirus, were later demonstrated to be encapsidated and produce infectious particles by using a recombinant vaccinia virus (VV-P1) that expresses the substituted poliovirus capsid precursor P1 proteins defective in the chimeric minireplicon (*J. Virol.* 67:3712, 1993). For use in accordance with this invention, the alphavirus vector genome is substituted for the P1 capsid sequences and used as a means for providing polio-pseudotyped alphavirus vectors after transfecting in vitro transcribed alphavirus vector RNA transcripts into the cell line. Conversely, alphavirus structural proteins also may be substituted for the VP2 and VP3 proteins, subsequently providing an alternative packaging cell line system for alphavirus based vectors.

In an alternative system, several components are used, including: (1) alphavirus structural proteins made in the baculovirus system using techniques described by Smith et

al. (supra) (or in other protein production systems, such as yeast or *E. coli*); (2) viral vector RNA made in the known T7, SP6 or other in vitro RNA-generating system (Flamant et al., *J. Virol.* 62:1827, 1988); (3) tRNA transcribed in vitro or purified from yeast or mammalian tissue culture cells; (4) liposomes (with embedded envelope glycoproteins); and (5) cell extract or purified necessary components when identified (typically from mouse cells) to provide RNA processing, and any or other necessary cell-derived functions.

Within this procedure, components (1), (2) and (3), from above, are mixed, and then envelope glycoprotein associated alphavirus proteins, cell extract and pre-liposome mix (lipid in a suitable solvent) are added. In an alteration of the procedure, the alphavirus envelope glycoproteins are embedded in the liposomes prior to addition to the mixture of (1), (2), and (3). The resulting mixture is then treated (e.g., by sonication, temperature manipulation, or rotary dialysis) to allow envelopment of the viral nucleocapsid particles with lipid plus embedded alphavirus envelope glycoprotein in a manner similar to that for liposome encapsidation of pharmaceuticals (Gould-Fogerite et al., *Anal. Biochem.* 148:15, 1985). This or similar procedures can be used to produce high titres of packaged alphavirus vectors without the requirement of establishing intermediate packaging cell lines.

#### Example 9

##### CELL LINE OR TISSUE SPECIFIC ALPHAVIRUS VECTORS-"HYBRID ENVELOPES"

The tissue and cell-type specificity of alphaviruses is determined primarily by the virus-encoded envelope proteins, E1 and E2. These virion structural proteins are transmembrane glycoproteins embedded in a host cell-derived lipid envelope that is obtained when the viral particle buds from the surface of the infected cell. The envelope surrounds an icosahedral nucleocapsid, comprised of genomic RNA complexed with multiple, highly ordered copies of a single capsid protein. The E1 and E2 envelope glycoproteins are complexed as heterodimers which have been reported to assemble into trimeric structures, forming the characteristic "spikes" on the virion surface. In addition, the cytoplasmic tails of these proteins interact with the nucleocapsids, initiating the assembly of new viral particles (*Virology* 193:424, 1993). Properties ascribed to the individual glycoproteins of Sindbis virus include receptor binding by glycoprotein E2 (*Virology* 181:694, 1991) and glycoprotein E1-mediated fusion of the virion envelope and the endosomal membrane, resulting in delivery of the nucleocapsid particle into the cytoplasm (*New Aspects of Positive-Stranded RNA Virus*, pp. 166-172, 1990).

The present invention recognizes that by disrupting glycoprotein activity (in particular, but not limited to that of E2) and co-expressing an intact heterologous glycoprotein, or by creating hybrid envelope gene products (i.e., specifically, an alphavirus envelope glycoprotein having its natural cytoplasmic domain and membrane-spanning domain, with its exogenous binding domain replaced by the corresponding domain(s) from a different envelope glycoprotein, or by replacing the E2 and/or E1 glycoproteins with those of other alphaviruses or their derivatives which differ from that of the vector in their tissue tropism, the host range specificity may be altered without disrupting the cytoplasmic functions required for virion assembly. Alternatively, by replacing one or more of the alphavirus structural proteins with the struc-

tural protein(s) of another virus and introducing the corresponding viral packaging sequence into the alphavirus vector construct, assembly of recombinant alphavirus vector constructs into particles of other virus types can be achieved. Thus, recombinant alphavirus particles can be produced which have an increased affinity for pre-selected target cells, depending on the tropism of the protein molecule(s) or domain(s) introduced.

In one embodiment, substitution of the analogous envelope glycoproteins E1 and/or E2 from other alphaviruses or their variants is performed to alter tissue tropism. For example, Venezuelan equine encephalitis virus (VEE) is an alphavirus which exhibits tropism for cells of lymphoid origin, unlike its Sindbis virus counterpart. Therefore, Sindbis-derived vector constructs packaged in a cell line expressing the VEE structural proteins display the same lymphotropic properties as the parental VEE virus from which the packaging cell structural protein gene cassette was obtained.

Specifically, the Trinidad donkey strain of VEE virus (ATCC #VR-69) is propagated in BHK-21 cells, and virion RNA is extracted using procedures similar to those described in Example 1. The entire structural protein coding region is amplified with a primer pair whose 5'-ends map, respectively, to the authentic AUG translational start site, including the surrounding Kozak consensus sequence, and UGA translational stop site. The forward primer is complementary to VEE nucleotides 7553-7579, and the reverse primer is complementary to VEE nucleotides 11206-11186 (sequence from Kinney et al., *Virology* 170:19-30, 1989). PCR amplification of VEE cDNA corresponding to the structural protein genes is accomplished using a two-step reverse transcriptase-PCR protocol as described above, the VEE genome RNA as template, and the following oligonucleotide pair:

Forward primer (VEE 7553F):

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5'-TATATATATGCGGCGCACCGCCAAGATGTTCCGTTCCAG-  
CCA-3' (SEQ. ID NO. 90)

---

Reverse primer (VEE 11206R):

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5'-TATATATATGCGGCGCTCAATTATGTTTCTGGTTGGT-3'  
(SEQ. ID NO. 91)

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In addition to their respective complementarities to the indicated VEE nucleotides, each primer includes a Not I recognition sequence at their 5' ends. Following PCR amplification, the 3800 bp fragment is purified in a 1% agarose gel and digested with the enzyme Not I. The resulting fragment is then ligated separately into the pOP13 and pOPRSV1 vectors (Stratagene) described previously, which are digested with Not I and treated with calf intestinal alkaline phosphatase. These resulting vectors, which contain the entire VEE structural protein coding sequence, are known as pOP13-VEESP and pOPRSV1-VEESP. The use of these clones in the development of VEE-based packaging cell lines follows that described for Sindbis packaging lines. Alternatively, the PCR amplified VEE structural protein gene fragment digested with NotI is ligated into the replicon inducible ELVIS cassette described in Example 7. Plasmid pVGELVISBV-linker is digested with Bsp EI to remove most nonstructural protein coding sequences, and the vector is then re-ligated with itself to generate the construct pVGELVISd1-linker. Subsequently, this plasmid is digested

with NotI, treated with calf intestinal alkaline phosphatase, and ligated with the NotI digested VEE fragment to generate the expression cassette pVGELVd1VEE. Plasmid DNA of this construct is transfected into the appropriate cell line and selection for G418 resistance is performed as described in Example 7. In addition, variations of the vector-inducible or lac operon-VEE structural protein gene expression vectors may be constructed using other systems described herein. Additionally, other variations may be constructed which combine the capsid protein gene of one alphavirus (for example, Sindbis) with the envelope glycoprotein genes of another alphavirus (for example, VEE) in a split gene approach, as described in Example 7. Furthermore, variants of VEE, and other alphaviruses and their variants differing in tissue tropism, are useful when following this approach.

In another embodiment, a RNA packaging signal derived from another virus is inserted into the alphavirus vector to allow packaging by the structural proteins of that corresponding virus. For example, the 137 nt. packaging signal from hepatitis B virus, located between nts. 3134 and 88 and spanning the precore/core junction (Junker-Niepmann et al. *EMBO J.* 9:3389, 1990), is amplified from an HBV template using two oligonucleotide primers. PCR is performed using a standard three temperature cycling protocol, plasmid pHBV1.1 (Junker-Niepmann et al. *EMBO J.* 9:3389, 1990) as the template, and the following oligonucleotide pair, each of which contain 20 nucleotides complementary to the HBV sequence and flanking Apal recognition sequences:

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5'-TATATGGGCCCTACATGTCCCACTGTTCAAG-3'  
(SEQ. ID NO. 117)

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Reverse primer (HBVpkgR):

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5'-TATATGGGCCCGTACGGAAGGAAAGAAGTCA-3'  
(SEQ. ID NO. 118)

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Following amplification, the PCR amplicon is digested with Apal and purified from a 1.5% agarose gel using MERMAID™ (Bio101). Sindbis vector plasmid pKSSINd1JRsrc (Example 3) also is digested with Apal, under limited conditions to cleave at only one of its two sites, followed by treatment with CIAP, purification from a 1% agarose gel, and ligation with the above-synthesized HBV amplicon, to produce a construct designated pKSSIN-hbvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Cell lines which express the HBV core, preS/S, and P proteins necessary for packaging of the RNA sequence are derived by modification of helper plasmid pCH3143 (Junker-Niepmann et al., *EMBO J.* 9:3389, 1990) to include a selectable marker. An expression cassette containing the neomycin resistance marker is obtained by digestion of plasmid pBK-RSV (Stratagene) with Mst II and blunt-ending with Klenow fragment. The selectable marker is then ligated into any one of several unique sites within pCH3143 that have been digested and their termini made blunt. The resulting construct is transfected into a desired cell line, for example, mouse hepatoma line Hepa1-6 (ATCC #CRL1830), and selected using the drug G418, as described in Example 7. Introduction of the pKSSINhbvJR vector, or related RNA- or DNA-based alphavirus vectors, results in the production of packaged vector particles with the same hepatotropism as HBV.

Similarly, the packaging signal from a coronavirus can be incorporated into the alphavirus vector. For example, the

190 nt packaging signal from mouse hepatitis virus (MHV), comprising nts 2899 to 3089 (Fosmire et al., *J. Virol.* 66:3522, 1992), is amplified in a standard three cycle PCR protocol using TBERMALASE™ polymerase, D1ssF plasmid MP51-2 (Fosmire et al., *J. Virol.* 66:3522, 1992) as the template, and the following oligonucleotides, which contain flanking Apal recognition sites:

Forward primer (MHVpkgF):

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5'-TATATGGGCCCATTTTGGTTTGGCTATGCGTA-3'  
(SEQ. ID NO. 119)

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Reverse primer (MHVpkgR):

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5'-TATATGGGCCCATCGAGGTGAGAAAGAGGAC-3'  
(SEQ. ID NO. 125)

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Following amplification, the PCR amplicon is digested with Apal, purified from a 1.5% agarose gel using MERMAID™, and ligated into pKSSINd1JRsrc, prepared as described for HBV. The resulting construct is designated pKSSINmhvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Packaging of vectors modified with this MHV sequence is accomplished by using expression cassettes which produce each of the required coronavirus structural proteins: nucleocapsid (N protein; Armstrong et al., *NAR* 11:883, 1983); membrane (M protein; Armstrong et al., *Nature* 308:751, 1984); and spike (S protein; Luytjes et al., *Virology* 161:479, 1987). Preferably, these proteins are inserted into the vector-inducible pVGELVsd1-linker plasmid (described previously in this example) and selected for with the G418 drug following transfection into the appropriate cell type. Other expression methodologies (see Example 7) may also be readily utilized. Additional coronaviruses, for example, human coronaviruses OC43 (ATCC #VR-759) and 229E (ATCC #VR-740), can be readily used in place of MHV to produce packaged recombinant alphavirus particles which show tropism for cells in the respiratory tract.

Similarly, the packaging signal from a retrovirus can be incorporated into an alphavirus vector construct. For example, the 351 nt extended packaging signal (ψ+) from Mo-MLV, corresponding to nts 212 to 563 (Mann et al., *Cell* 33:153, 1983), is amplified in a standard three cycle PCR protocol as described above, using plasmid pMLV-K (Miller, *J. Virol.* 49:214, 1984) as template and the following oligonucleotides, each of which contain a flanking Apal recognition site:

Forward primer (MLVpkgF):

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5'-TATATGGGCCCTGTATCTGGCGGACCGTGG-3'  
(SEQ. ID NO. 126)

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Reverse primer (MLVpkgR):

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5'-TATATGGGCCCGCAGACAAGACGCGGGCGC-3'  
(SEQ. ID NO. 127)

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Following amplification, the PCR amplicon is digested with Apal, purified from a 1.5% agarose gel using GENE CLEAN™, and ligated into plasmid pKSSINd1JRsrc, prepared as described above. The resulting construct is designated pKSSINm1vJR. Other alphavirus vectors (see Example 3) are readily modified in a similar



manner. The generation of a retroviral-derived producer cell line for packaging and production of the above alphavirus vector constructs is accomplished by transfecting an appropriate packaging cell line, for example amphotropic line DA (WO 92/05266), and selecting for resistance to the drug G418, as described previously.

In each case, the packaging sequences from HBV, coronavirus, retrovirus, or any other virus, also may be incorporated into alphavirus vectors at locations other than those outlined above, provided the location is not present in the subgenomic transcript. For example, the next most preferable site of insertion is the carboxy-terminal region of nonstructural protein 3, which has been shown to be highly variable in both length and sequence among all alphaviruses for which sequence information is available. Further, these applications are not limited by the ability to derive the corresponding packaging cell lines, as the necessary structural proteins also may be expressed using any of the alternative approaches described in Example 8.

In yet another embodiment, a heterologous glycoprotein or cellular ligand is expressed in the lipid bilayer of a packaging cell line for producing enveloped recombinant alphavirus particles. This approach is similar to that described in Example 8 for the production of VSV-G pseudo-typed alphavirus vectors, except that in this configuration, the E2 receptor-binding function is inactivated by insertion, deletion, or site-specific mutagenesis. As an example, receptor binding function of E2 can be inactivated by techniques known in the art to restrict vector particle tropism to that which is supplied by the heterologous glycoprotein or cellular ligand. In addition to the example of VSV-G pseudo-typing, other viral glycoproteins which target specific cellular receptors (such as the retroviral HIV gp120 protein for CD4 cell targeting) are utilized when expressed from standard vectors stably transfected into alphavirus packaging cell lines.

In another configuration, chimeric glycoproteins are prepared which allow for targeting of alphavirus vector constructs into particular cell lines in vitro or tissue types in vivo. To construct such a chimeric glycoprotein, specific oligonucleotides encoding the ligand binding domain of the desired receptor, plus homologous alphavirus sequences (which include a unique specific restriction endonuclease site), are used to amplify an insert sequence that can be substituted into an alphavirus structural protein expression cassette. Alternatively, limited Bal-31 digestions from a convenient restriction enzyme site are performed in order to digest back to a permissive insertion site, followed by blunt end ligation of a fragment encoding a small receptor binding domain, an entire viral glycoprotein, or cell surface ligand. As an example, peptides corresponding to the principal neutralizing domain of the HIV gp120 envelope protein (*Virology* 185:820, 1991) can be used to disrupt normal E2 tropism and provide CD4 cell targeting.

While inclusion of the HIV gp120 neutralizing domain illustrates one example of a hybrid or chimeric envelope protein, the possibilities are not limited to viral glycoproteins. For example, the receptor binding portion of human interleukin-2 can be combined with the envelope protein(s) of an alphavirus to target vectors to cells with IL-2 receptors. Furthermore, the foregoing technique can be used to create a recombinant alphavirus particles with envelope proteins that recognize Fc portions of antibodies. Monoclonal antibodies which recognize only preselected target cells are then bound to such Fc receptor-bearing alphavirus vector particles, such that the vector particles bind to and infect only those preselected target cells (for example, tumor

cells). Alternatively, a hybrid envelope with the binding domain of avidin is used to target cells that have been coated with biotinylated antibodies or other ligands. The patient is first flooded with antibodies, and then allowed time to clear unbound and nonspecifically-bound antibody before administering the vector. The high affinity ( $10^{-15}$ ) of the avidin binding site for biotin will allow accurate and efficient targeting to the original tissue identified by the monoclonal "image". Additional targeting approaches are known in the art and can readily be adopted for use in the practice of the present invention. For example, see U.S. Ser. No. 08/242,407.

#### Example 10

##### LACTOSE FORMULATION OF A RECOMBINANT ALPHAVIRUS VECTOR

Crude recombinant alphavirus particles are obtained from a Celligen bioreactor (New Brunswick, N.J.) containing packaging cells transfected or transduced with the alphavirus vector construct, and bound to the beads of the bioreactor matrix. The cells release the packaged recombinant alphavirus particles into growth media that is passed over the cells in a continuous flow process. The media exiting the bioreactor is collected and passed initially through a 0.8 micron filter, then through a 0.65 micron filter to clarify the crude recombinant alphavirus particles. The filtrate is concentrated utilizing a cross flow concentrating system (Filtron, Boston, Mass.). Approximately 50 units of DNase (Intergen, New York, N.Y.) per ml of concentrate is added to digest exogenous DNA. The digest is diafiltered using the same cross flow system to 150 mM NaCl, 25 mM tromethamine, pH 7.2. The diafiltrate is loaded onto a Sephadex S-500 gel column (Pharmacia, Piscataway, N.J.), equilibrated in 50 mM NaCl, 25 mM tromethamine, pH 7.4. The purified recombinant alphavirus particles are eluted from the Sephadex S-500 gel column in 50 mM NaCl, 25 mM tromethamine, pH 7.4.

The formulation buffer containing lactose is prepared as a 2x concentrated stock solution. The formulation buffer contains 25 mM tromethamine, 70 mM NaCl, 2 mg/ml arginine, 10 mg/ml human serum albumin (HSA), and 100 mg/ml lactose in a final volume of 100 mls at a pH 7.4.

The purified recombinant alphavirus particles are formulated by adding one part 2x lactose formulation buffer to one part S-500 purified recombinant alphavirus particle preparation. The formulated recombinant alphavirus particles can be stored at  $-70^{\circ}\text{C}$ . to  $-80^{\circ}\text{C}$ . or dried.

The formulated alphavirus particles are lyophilized in an Edwards Refrigerated Chamber (3 Shelf RC3S unit) attached to a Supermodulyo 12K freeze dryer (Edwards High Vacuum, Tonawanda, N.Y.). When the freeze drying cycle is completed, the vials are stoppered under a vacuum following a slight nitrogen gas bleeding. Upon removal, vials are crimped with aluminum seals. The lyophilized recombinant alphavirus particles are reconstituted with 1.0 ml water or other physiologically acceptable diluent.

#### Example 11

##### ADMINISTRATION OF RECOMBINANT ALPHAVIRUS PARTICLES

A therapeutic alphavirus vector used for the treatment of Gaucher disease (see Example 17) may be administered by transducing autologous CD34<sup>+</sup> cells in an ex vivo protocol or by direct injection of the vector into the patient's bone

marrow. In order to achieve the longest therapeutic expression of GC from the recombinant multivalent vector, the best mode of administration is to transduce long lived cell precursors of the clinically affected cell type, for example monocytes or macrophages. By transducing the earliest precursors of the effected cell type, the cell precursors are able to self renew and repopulate the peripheral blood with maturing GC positive cells. The earliest pluripotent hematopoietic stem cell studied to date are the CD34<sup>+</sup> cells which make up 1%–4% of a healthy bone marrow population or 0.1% in the peripheral blood population. Being able to transduce CD34<sup>+</sup> cells is important in sustaining long term expression not only for the monocyte/macrophage lineage but any hematopoietic cell targeted for a therapeutic protein. Two approaches for transducing CD34<sup>+</sup> cells include an ex vivo and an in vivo protocol. The in vivo protocol focuses on transducing an indiscriminate population of bone marrow cells by direct injection of the vector into the bone marrow of patients. The ex vivo protocol focuses on isolating CD34<sup>+</sup> positive stem cells, from the patient's bone marrow, or an infant patient's umbilical cord blood, transducing the cells with vector, then subsequently injecting the autologous cells back into the patient. Both approaches are feasible, but the ex vivo protocol enables the vector to be used most efficiently by transducing a specific cultured population of CD34<sup>+</sup> cells. Details of an ex vivo method are provided in the following section.

#### EX VIVO ADMINISTRATION OF A MULTIVALENT GC SINDBIS VECTOR

CD34<sup>+</sup> cells are collected from the patient's bone marrow by a syringe evacuation performed by a physician familiar with the technique. Alternatively, CD34<sup>+</sup> cells may also be obtained from an infant's umbilical cord blood if the patient is diagnosed before birth. Generally, if the bone marrow is the source of the CD34<sup>+</sup> cells, 20 bone marrow aspirations are obtained by puncturing femoral shafts or from the posterior iliac crest under local or general anesthesia. Bone marrow aspirations are then pooled, suspended in HEPES-Buffered Hanks' balanced salt solution containing heparin at 100 units per ml and deoxyribonuclease I at 100 ug/ml and then subjected to a Ficoll gradient separation. The buffy coated marrow cells are then collected and washed according to CeliPro's CEPRATE® LC (CellPro, Bothell, Wash.) (CD34) Separation system (see U.S. Pat. Nos. 5,215,927; 5,225,353; 5,262,334; 5,215,926 and PCT/US91/07646). The washed buffy coated cells are then stained sequentially with anti-CD34 monoclonal antibody, washed then stained with biotinylated secondary antibody supplied with CEPRATE® system. The cell mixture is then loaded onto the CEPRATE® avidin column. The biotin-labeled cells are adsorbed onto the column while unlabeled cells passed through. The column is then rinsed according to the CEPRATE® system directions and CD34<sup>+</sup> cells eluted by agitation of the column by manually squeezing the gel bed. Once the CD34<sup>+</sup> cells are purified, the purified stem cells are counted and plated at a concentration of  $1 \times 10^5$  cells/ml in Iscove's modified Dulbecco's medium (IMDM; Irvine Scientific, Santa Ana, Calif.) containing 20% pooled non-heat inactivated human AB serum (hAB serum).

After purification, several methods of transducing purified stem cells may be performed. One approach involves immediate transduction of the purified stem cell population with recombinant alphavirus particles contained in culture supernatants derived from vector packaging or producing cells. A second approach involves co-cultivation of an irradiated monolayer of vector producing cells with the purified popu-

lation of nonadherent CD34<sup>+</sup> cells. A third approach involves a similar co-cultivation approach, however, the purified CD34<sup>+</sup> cells are prestimulated with various cytokines and cultured 48 hours prior to the co-cultivation with the irradiated vector producing cells. Since alphavirus vectors are able to infect nonreplicating cells, prestimulation of these cells may not be required, however prestimulation of these cultures causing proliferation will provide increased cell populations for reinfusion into the patient.

Prestimulation of the CD34<sup>+</sup> cells is performed by incubating the cells with a combination of cytokines and growth factors which include IL-1, IL-3, IL-6 and mast cell growth factor (MGF). Prestimulation is performed by culturing  $1-2 \times 10^5$  CD34<sup>+</sup> cells/ml of medium in T25 tissue culture flasks containing bone marrow stimulation medium for 48 hours. The bone marrow stimulation medium consists of IMDM containing 30% non-heat inactivated hAB serum, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 1M hydrocortisone, and 1% deionized bovine serum albumin. All reagents used in the bone marrow cultures should be screened for their ability to support maximal numbers of granulocyte, erythrocyte, macrophage, megakaryocyte, colony-forming units from normal marrow. Purified recombinant human cytokines and growth factors (Immunex Corp., Seattle, Wash.) for prestimulation should be used at the following concentrations: *E. coli*-derived IL-1 (100 U/ml), yeast-derived IL-3 (5 ng/ml), IL-6 (50 U/ml), and MGF (50 ng/ml) (Anderson et al., *Cell Growth Differ.* 2:373, 1991).

After prestimulation of the CD34<sup>+</sup> cells, they are then infected by co-cultivation with the irradiated Sindbis producer cell line (expressing the GC therapeutic vector) in the continued presence of the stimulation medium. The Sindbis vector producing cell line is first trypsinized, irradiated (10,000 Rads) and replated at  $1-2 \times 10^5$  cells/ml of bone marrow stimulation medium. The following day,  $1-2 \times 10^5$  prestimulated CD34<sup>+</sup> cells/ml is added to the Sindbis vector producing cell line monolayer. Co-cultivation of the cells is performed for 48 hours. After co-cultivation, the CD34<sup>+</sup> cells are collected from the adherent Sindbis vector producing cell monolayer by vigorous washing with medium and plated for 2 hours to allow adherence of any dislodged vector producing cells. The CD34<sup>+</sup> cells are collected and expanded for an additional 72 hours. The cells are then harvested and frozen in liquid nitrogen using a cryoprotectant in aliquots of  $1 \times 10^7$  cells per vial. Once the treated CD34<sup>+</sup> cells have been tested for the absence of adventitious agents, frozen transformed CD34<sup>+</sup> cells may be thawed, plated to a concentration of  $1 \times 10^5$  cells/ml and cultured for an additional 48 hours in bone marrow stimulation medium. Transformed cells are collected, washed twice and resuspended in normal saline. The number of transduced cells used to infuse back into the patient per infusion is projected to be at a minimum of  $1-10 \times 10^7$  cells per patient per injection site requiring up to four injection sites. Infusion may be performed directly back into the patient's bone marrow or directly into the peripheral blood stream. Patients receiving autologous transduced bone marrow cells may be either partially or whole body irradiated, to deplete existing bone marrow populations. Treatment may be assessed at various time points post infusion to determine GC activity and for length of expression in differentiated cell types. If at some point during the course of follow-up procedures expression decreases or is nonexistent, transduced autologous cells may be reinjected into the patient.

# DETERMINATION OF VECTOR UNITS IN A PREPARATION BY INFECTION OF A REPORTER PROTEIN EXPRESSING CELL LINE UNDER THE CONTROL OF THE SINDBIS JUNCTION REGION

## DETERMINATION OF VECTOR UNITS IN A PREPARATION BY INFECTION OF A $\beta$ - GALACTOSIDASE EXPRESSING REPORTER CELL LINE

In order to administer the proper therapeutic dose of vector to individuals, it is desirable to derive a method by which the vector infectious units contained in a preparation can be determined easily. This is accomplished by the generation of a cell line which expresses  $\beta$ -galactosidase or another reporter gene only when functional Sindbis non-structural proteins are present in the cell. The cell line can be infected with increasing dilutions of a Sindbis vector preparation such that individual cells are not infected with more than one vector particle, allowing the titer, or vector units, to be determined. Thus, the cell line is an assay of functional particles present in a vector preparation.

### A. GENERATION OF A CELL LINE WHICH EXPRESSES FUNCTIONAL $\beta$ -GALACTOSIDASE PRO- TEIN UNDER THE CONTROL OF SINDBIS NON- STRUCTURAL PROTEINS

In one configuration, a eukaryotic expression cassette is constructed which contains a 5'-end sequence capable of initiating transcription of Sindbis RNA, a Sindbis junction region, a reporter gene, and a 3'-end Sindbis RNA polymerase recognition sequence for minus-strand synthesis. This cassette is positioned in an antisense orientation, adjacent to a eukaryotic transcriptional promoter. Additionally, these constructs also may contain a catalytic ribozyme sequence immediately adjacent to Sindbis nucleotide 1 of the 5'-end sequence which will result in cleavage of the primary RNA transcript precisely after this Sindbis nucleotide. In this antisense orientation, the reporter gene cannot be translated and is dependent entirely on the presence of Sindbis nonstructural proteins for transcription into positive stranded mRNA prior to reporter gene expression. These non-structural proteins will be provided by the Sindbis vector preparation being titered. In addition, this configuration, if designed to contain the precise Sindbis genome 5'- and 3'-end sequences, will allow for the reporter gene transcripts to undergo amplification by utilizing the same nonstructural proteins provided by the Sindbis vector.

An example of this antisense titration construction is as follows. Briefly, the plasmid pKSSINBV-lacZ (described in Example 6) is digested with the enzymes Apa I and Bam HI. This results in the removal of the Sindbis 5' and Sindbis nonstructural protein sequences. The 7 kbp fragment is purified on a 0.7% agarose gel. This fragment is ligated to a fragment obtained by digestion of pd5'26s (described in Example 7) with ApaI and BamHI followed by gel purification of the 0.4 kbp fragment containing the HDV ribozyme and 5' Sindbis sequences. The resulting construct is known as pKSd5'BV-lacZ. pKSd5'BV-lacZ is digested with Apa I and Pme I followed by purification of the 7.4 kbp fragment on a 0.7% agarose gel. This fragment contains the HDV ribozyme, Sindbis 5' end, junction region, LacZ gene, and Sindbis 3' end sequences. This fragment is ligated in the antisense orientation into pcDNA3 (Promega Corp., Madison, Wis.) by digestion of pcDNA3 with Apa I and EcoRV followed by GENECLAN™ purification. The

resulting construct, containing a CMV promoter which transcribes an antisense reporter cassette RNA of the configuration Sindbis 3'-end sequence/LacZ gene/junction region/Sindbis 5'-end sequence/HDV ribozyme, is known as pSINjra-gal.

BHKSINjra-gal cells are derived by transfection of  $5 \times 10^5$  BHK-21 cells, grown in a 60 mm petri dish, with 5  $\mu$ g of the pSINjra-gal vector complexed with the polycation reagent Transfectam™ (Promega, Madison, Wis.). At 24 hour post-transfection, the media is supplemented with 400  $\mu$ g/ml of G418 (GibcoBRL, Gaithersburg, Md.). After all non-transfected cells have died and G418 resistant colonies have begun dividing, the cells are removed from the plate by trypsinization, pooled, then cloned by limiting dilution. Several clones are tested for the production of functional  $\beta$ -galactosidase by infection with a known titer of a wild-type stock of Sindbis virus. Production of functional  $\beta$ -galactosidase in candidate BHKSINjra-gal clones is determined 6 hours post-infection by first fixing PBS-rinsed cells with a solution containing 2% formaldehyde (37% stock solution)/0.2% glutaraldehyde, then staining the cells with a solution containing 0.5 mM potassium ferricyanide/0.5 mM potassium ferrocyanide/2 mM  $MgCl_2$ /1 mg/ml X-gal. Blue cells are clearly visible within 3 hours. Provided that the Sindbis virus stock does not contain a high level of defective interfering (DI) particles, the virus titer as determined by plaque assay on BHK-21 cells should be similar to the titer observed by X-gal staining on BHKSINjra-gal cells.

The titer of various alphavirus vector preparations, in vector units, produced from packaging cell lines such as those described in Example 7, is determined by infection of confluent monolayers of BHKSINjra-gal cells with several dilutions of vector. The titer of the vector preparation is determined at 6 hour post-infection by visualization of cells producing  $\beta$ -galactosidase protein, as described above. Since the alphavirus vectors described do not contain the viral region corresponding to the structural genes, it is not possible to determine the titer of a vector preparation by plaque assay in BHK-21 cells.

Alternatively, a titrating cell line is produced by using a different reporter cassette configuration, which consists of a eukaryotic promoter/5'-end Sindbis sequence recognized by the viral transcriptase/Sindbis junction region/reporter gene/Sindbis RNA polymerase recognition sequence for minus-strand synthesis, and is expressed in a sense-orientation. This reporter expression cassette requires synthesis, by vector-supplied Sindbis nonstructural proteins, into an antisense RNA molecule, prior to transcription of the subgenomic message encoding the reporter gene.

Specifically, the sense-orientation packaging construct is created as follows. Plasmid pVGELVIS is digested with the enzyme Apa I, which cleaves at nucleotide 11737, just downstream of the Sindbis 3'-end. The Apa I-digested DNA is blunt-ended by the addition of T4 DNA polymerase and dNTPs and incubation at 16° C. for 10 minutes. After heat inactivation of the polymerase, the DNA fragment is digested with the enzyme Sfi I, and the 10041 bp fragment is purified in a 1% agarose gel. Plasmid pKSINBV-lacZ is digested with the enzymes Pme I and Sfi I. The 6.4 kbp fragment is purified in a 1% agarose gel. The 6.4 kbp pKSINBV-lacZ fragment then is ligated into the purified pVGELVIS fragment to create the plasmid pELVIS-gal. This plasmid contains the complete Sindbis nonstructural proteins, Sindbis junction region, LacZ gene and Sindbis 3'-end replicase recognition sequence under the control of the MuLV LTR promoter. Plasmid pELVIS-gal is digested with Bsp EI, purified by GENECLAN (Bio 101 Corp., San

Diego, Calif.) and religated to itself. Bsp E1 removes the Sindbis nonstructural protein gene sequences between nts 422-7054. The re-ligated construct contains a 5' sequence that is capable of initiating transcription of Sindbis RNA, Sindbis junction region, sequences encoding the LacZ gene, and Sindbis 3'-end sequences required for synthesis of the minus-strand RNA, all downstream, and under the transcriptional control of a MuLV-LTR promoter. This construct is known as pELVISd1NSP-gal.

Plasmid pELVISd1NSP-gal is transfected into BHK-21 cells and tested as described previously. The BHK pELVISd1NSP-gal cells produces an RNA transcript with a 5'-end sequence that is recognized by the Sindbis transcriptase, a Sindbis junction region, sequences encoding the LacZ gene, and Sindbis 3'-end sequences required for synthesis of the minus-strand RNA.  $\beta$ -galactosidase expression from the primary transcript is prevented because of an upstream open-reading frame and stop codons created by the Bsp E1 deletion. The addition of Sindbis nonstructural proteins, provided by the Sindbis vector being titered, results in transcription of active LacZ transcripts from the Sindbis junction region, after initial synthesis of an antisense intermediate. Furthermore, this configuration, if designed to contain the precise Sindbis genome 5'- and 3'-end sequences, allows the reporter gene transcripts to undergo amplification by utilizing the same nonstructural proteins provided by the Sindbis vector.

In another configuration, a titrating cell line is produced using an expression cassette containing an antisense reporter gene followed by the 3'-end alphavirus replicase recognition sequences, positioned in the sense-orientation. This construct, under the control of a eukaryotic promoter, produces an RNA transcript that is recognized and transcribed by alphavirus nonstructural proteins provided by the vector to be titered. The alphavirus nonstructural proteins recognize sequences in the primary reporter transcript, and in turn, synthesize a sense reporter transcript. This construct does not benefit from amplification of the reporter gene transcript, but should still provide sufficient transcripts to allow for vector titrating.

Construction of this type of titrating cassette is as follows. Briefly, pSV- $\beta$ -galactosidase vector (Promega Corp., Madison, Wis.) is digested with the enzyme Hind III and blunt-ended as described above. The plasmid is further digested with the enzymes Bam HI and Xmn I to remove the LacZ gene, and reduce the size of the remaining fragment. The 3737 nt fragment, containing the LacZ gene, is purified in a 1% agarose gel and ligated into pcDNA3 (Invitrogen, San Diego, Calif.) that has been digested with the enzymes Bam HI and Eco RV. The new plasmid construct is known as pcDNAaLacZ. This plasmid is digested with the enzyme Apa I, blunt-ended as above, and further digested with the enzyme Xho I. Plasmid pSKSINBV (described previously) is digested with Sac I, blunt-ended as before, and then digested with Xho I. The resulting 146 nt fragment containing the Sindbis 3' replicase recognition sequence is purified in a 1.2% agarose gel, ligated into the digested pcDNAaLacZ vector. The re-ligated construct contains an antisense LacZ gene and a 3' Sindbis replicase protein recognition sequence downstream from a CMV promoter. The resulting construct is known as pcDNAaLacZ-3'Sin. The construct is transfected into BHK cells and utilized as described previously.

**B . GENERATION OF A CELL LINE WHICH EXPRESSES FUNCTIONAL LUCIFERASE PROTEIN UNDER THE CONTROL OF SINDBIS NONSTRUCTURAL PROTEINS.**

An alternate reporter for a titrating construct based upon the sense configuration of the reporter gene and requiring the nonstructural proteins for expression utility is luciferase. Again, the non-structural proteins are supplied in trans by the Sindbis vector preparation being titered. To generate this construct, pELVIS-luc is digested with Eco 47 III and Hpa I. These digests remove nucleotides 1407-6920 from within the non-structural coding region. After heat inactivation of the enzymes, the digested vector is religated under dilute conditions. This construct is known as pELVISd1E-H1uc. The construct is transfected into BHK cells and utilized as described previously.

### Example 13

#### GENERATION OF VECTOR CONSTRUCTS WHICH EXPRESS HBV ANTIGENS FOR THE INDUCTION OF AN IMMUNE RESPONSE

##### A. ISOLATION OF HBV E/CORE SEQUENCE

A 1.8 Kb fragment containing the entire precore/core coding region of hepatitis B is obtained from plasmid pAM6 (ATCC No 45020) following Bam HI digestion and gel purification, and ligated into the Bam HI site of KS II+ (Stratagene, La Jolla, Calif.). This plasmid is designated KS II+ HBpc/c. Xho I linkers are added to the Stu I site of precore/core in KS II+ HBpc/c (at nucleotide sequence 1,704), followed by cleavage with Hinc II (at nucleotide sequence 2,592). The resulting 877 base pair Xho I-Hinc II precore/core fragment is cloned into the Xho I/Hinc II site of SK II+. This plasmid is designated SK+HBc.

##### B . PREPARATION OF SEQUENCES UTILIZING PCR

###### 1. SITE-DIRECTED MUTAGENESIS OF HBV E/CORE SEQUENCE UTILIZING PCR

The precore/core gene in plasmid KS II+ HB pc/c is sequenced to determine if the precore/core coding region is correct. This sequence was found to have a single base-pair deletion which causes a frame shift at codon 79 that results in two consecutive in-frame TAG stop codons at codons 84 and 85. This deletion is corrected by PCR overlap extension (Ho et al., *Gene* 77:51, 1989) of the precore/core coding region in plasmid SK+ HBc. Four oligonucleotide primers are used for the 3 PCR reactions performed to correct the deletion.

The first reaction utilizes two primers. The sense primer sequence corresponds to the nucleotide sequence 1,805 to 1,827 of the adw strain and contains two Xho I restriction sites at the 5' end. The nucleotide sequence numbering is obtained from Genbank (Intelligenics, Inc., Mountain View, Calif.).

---

5' CTC GAG CTC GAG CCA CCA GCA CCA TGC AAC TTT TT-3'  
(SEQ. ID NO. 92)

---

The second primer sequence corresponds to the anti-sense nucleotide sequence 2,158 to 2,130 of the adw strain of hepatitis B virus, and includes codons 79, 84 and 85.

---

5'-CTA CTA GAT CCC TAG ATG CTG GAT CTT CC-3'  
(SEQ. ID NO. 93)

---

The second reaction also utilizes two primers. The sense primer corresponds to nucleotide sequence 2,130 to 2,158 of the adw strain, and includes codons 79, 84 and 85.

---

5'-GGA AGA TCC AGC ATC TAG GGA TCT AGT AG-3'  
(SEQ. ID NO. 94)

---

The second primer corresponds to the anti-sense nucleotide sequence from SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop codon of the HBV precore/core coding region.

---

5'-GGG CGA TAT CAA GCT TAT CGA TAC CG-3'  
(SEQ. ID NO. 95)

---

The third reaction also utilizes two primers. The sense primer corresponds to nucleotide sequence 5 to 27 of the adw strain, and contains two Xho I restriction sites at the 5' end.

---

5'-CTC GAG CTC GAG GCA CCA GCA CCA TGC AAC TTT TT  
(SEQ. ID NO. 92)

---

The second primer sequence corresponds to the anti-sense nucleotide sequence from the SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop codon of the HBV precore/core coding region.

---

5'-GGG CGA TAT GAA GCT TAT GCA TAC CG-3'  
(SEQ. ID NO. 96)

---

The first PCR reaction corrects the deletion in the anti-sense strand and the second reaction corrects the deletion in the sense strands. PCR reactions one and two correct the mutation from CC to CCAV which occurs in codon 79 and a base pair substitution from TCA to TCT in codon 81. Primer 1 contains two consecutive Xho I sites 10 bp upstream of the ATG codon of HBV e coding region and primer 4 contains a Cla I site 135 bp downstream of the stop codon of HBV precore/core coding region. The products of the first and second PCR reactions are extended in a third PCR reaction to generate one complete HBV precore/core coding region with the correct sequence.

The PCR reactions are performed using the following cycling conditions: The sample is initially heated to 94° C. for 2 minutes. This step, called the melting step, separates the double-stranded DNA into single strands for synthesis. The sample is then heated at 56° C. for 30 seconds. This step, called the annealing step, permits the primers to anneal to the single stranded DNA produced in the first step. The sample is then heated at 72° C. for 30 seconds. This step, called the extension step, synthesizes the complementary strand of the single stranded DNA produced in the first step. A second melting step is performed at 94° C. for 30 seconds, followed by an annealing step at 56° C. for 30 seconds which is followed by an extension step at 72° C. for 30 seconds. This procedure is then repeated for 35 cycles resulting in the amplification of the desired DNA product.

The PCR reaction product is purified by 1.5% agarose gel electrophoresis and transferred onto NA 45 paper (Schleicher and Schuell, Keene, N.H.). The desired 787 bp DNA fragment is eluted from the NA 45 paper by incubating for 30 minutes at 65° C. in 400 l high salt buffer (1.5M NaCl, 20 mM Tris, pH 8.0, and 0.1 mM EDTA). Following elution, 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) is added to the solution. The mixture is vortexed and then centrifuged 14,000 rpm for 5 minutes in a Brinkmann

Eppendorf centrifuge (5415L). The aqueous phase, containing the desired DNA fragment, is transferred to a fresh 1.5 ml microfuge tube and 1.0 ml of 100% EtOH is added. This solution is incubated on dry ice for 5 minutes, and then centrifuged for 20 minutes at 10,000 rpm. The supernatant is decanted, and the pellet is rinsed with 500 l of 70% EtOH. The pellet is dried by centrifugation at 10,000 rpm under vacuum, in a Savant Speed-Vac concentrator, and then resuspended in 10 l deionized H<sub>2</sub>O. One microliter of the PCR product is analyzed by 1.5% agarose gel electrophoresis. The 787 Xho I-Cla I precore/core PCR amplified fragment is cloned into the Xho I-Cla I site of SK+ plasmid. This plasmid is designated SK+HBc-c. *E. coli* (DH5 alpha, Bethesda Research Labs, Gaithersburg, Md.) is transformed with the SK+HBc-c plasmid and propagated to generate plasmid DNA. The plasmid is then isolated and purified, essentially as described by Birnboim et al. (*Nuc. Acid Res.* 7:1513, 1979; see also *Molecular Cloning: A Laboratory Manual*, Sambrook et al. (eds.), Cold Spring Harbor Press, 1989). The SK+HBc-c plasmid is analyzed to confirm the sequence of the precore/core gene (FIG. 4).

## 2. ISOLATION OF HBV CORE SEQUENCE

The single base pair deletion in plasmid SK+HBc is corrected by PCR overlap extension as described above in Example 13B. Briefly, four oligonucleotide primers are used for the PCR reactions performed to correct the mutation.

The first reaction utilizes two primers. The sense primer corresponds to the nucleotide sequence for the T-7 promoter of SK+HBc plasmid.

---

5'-AAT ACG ACT CAC TAT AGG G-3'  
(SEQ. ID NO. 97)

---

The second primer corresponds to the anti-sense sequence 2,158 to 2,130 of the adw strain, and includes codons 79, 84 and 85.

---

5'-CTA CTA GAT CCC TAG ATG CTG GAT CTT CC-3'  
(SEQ. ID NO. 98)

---

The second reaction utilizes two primers. The anti-sense primer corresponds to the nucleotide sequence for the T-3 promoter present in SK+HBc plasmid.

---

5'-3': ATT AAC CCT CAC TAA AG  
(SEQ. ID NO. 99)

---

The second primer corresponds to the sense nucleotide sequence 2,130 to 2,158 of the adw strain, and includes codons 79, 84 and 85.

---

5'-GGA AGA TCC AGC ATC TAG GGA TCT AGT AG-3'  
(SEQ. ID NO. 100)

---

The third reaction utilizes two primers. The anti-sense primer corresponds to the nucleotide sequence for the T-3 promoter present in SK+HBc plasmid.

---

5'-ATT AAC CCT CAC TAA AG-3'  
(SEQ. ID NO. 101)

---

The second primer corresponds to the sense sequence of the T-7 promoter present in the SK+HBc plasmid.

---

5'-AAT ACG ACT CAC TAT AGG G-3'  
(SEQ. ID NO. 102)

---

The PCR product from the third reaction yields the correct sequence for HBV precore/core coding region.

To isolate HBV core coding region, a primer is designed to introduce the Xho I restriction site upstream of the ATG start codon of the core coding region, and eliminate the 29 amino acid leader sequence of the HBV precore coding region. In a fourth reaction, the HBV core coding region is produced using the PCR product from the third reaction and the following two primers.

The sense primer corresponds to the nucleotide sequence 1,885 to 1,905 of the adw strain and contains two Xho I sites at the 5' end.

---

5'-CCT CGA CGT CGA GCT TGG GTG GCT TTG GGG CAT G-3'  
(SEQ. ID NO. 103)

---

The second primer corresponds to the anti-sense nucleotide sequence for the T-3 promoter present in the SK+HBc plasmid. The approximately 600 bp PCR product from the fourth PCR reaction contains the HBV core coding region and novel Xho I restriction sites at the 5' end and Cla I restriction sites at the 3' end that was present in the multicloning site of SK<sup>+</sup> HBc plasmid.

---

5'-ATT ACC OCT CAC TAA AG-3'  
(SEQ. ID NO. 104)

---

Following the fourth PCR reaction, the solution is transferred into a fresh 1.5 ml microfuge tube. Fifty microliters of 3M sodium acetate is added to this solution followed by 500  $\mu$ l of chloroform:isoamyl alcohol (24:1). The mixture is vortexed and then centrifuged at 14,000 rpm for 5 minutes. The aqueous phase is transferred to a fresh microfuge tube and 1.0 ml 100% EtOH is added. This solution is incubated at -20° C. for 4.5 hours, and then centrifuged at 10,000 rpm for 20 minutes. The supernatant is decanted, and the pellet rinsed with 500  $\mu$ l of 70% EtOH. The pellet is dried by centrifugation at 10,000 rpm under vacuum and then resuspended in 10  $\mu$ l deionized H<sub>2</sub>O. One microliter of the PCR product is analyzed by 1.5% agarose gel electrophoresis. The approximately 600 bp Xho I-Cla I HBV core PCR fragment is cloned into the Xho I-Cla I site of SK<sup>+</sup> plasmid. This plasmid is designated SK+HBc.

### 3. ISOLATION OF HBV X ANTIGEN

A 642 bp Nco I—Taq I fragment containing the hepatitis B virus X open reading frame is obtained from the pAM6 plasmid (adw) (ATCC 45020), blunted by Klenow fragment, and ligated into the Hinc II site of SK<sup>+</sup> (Stratagene, La Jolla, Calif.). *E. coli* (DH5, Bethesda Research Laboratories, Gaithersburg, Md.) is transformed with the ligation reaction and propagated.

Since this fragment can be inserted in either orientation, clones are selected that have the sense orientation with respect to the Xho I and Cla I sites in the SK<sup>+</sup> multicloning site. More specifically, miniprep DNAs are digested with the diagnostic restriction enzyme, Bam HI. Inserts in the correct orientation yield two fragments of 3.0 Kb and 0.6 Kb in size. Inserts in the incorrect orientation yield two fragments of 3.6 Kb and 0.74 Kb. A clone in the correct orientation is selected and designated SK-X Ag.

### 4. CONSTRUCTION OF SINDBIS VECTORS EXPRESSING HBVE, HBV CORE AND HBV X

Construction of a Sindbis vector expressing the HBVe sequence is accomplished by digesting the SK<sup>+</sup> HB e-c plasmid with Xho I and Xba I to release the cDNA fragment encoding HBVe-c sequences. The fragment is then isolated by agarose gel electrophoresis, purified by GENECLEAN™, and inserted into pKSSINBV (see Example 3), prepared by digestion with Xho I and Xba I, and treated with CLAP. This vector is designated pKSSIN-HBe. Similar vectors may also be made from other Sindbis vectors described in Example 3, such as, for example, pKSSINd1JRsjrc, pKSSINd1JRsjrPC, pKSSINd1JRsjrNP (7582-7601) and pKSSINd1JRsexjr.

Construction of a Sindbis vector expressing the HBV core sequence is accomplished by digestion of plasmid SK+HBc (described above) with Xho I and Xba I. The HBc fragment is isolated by agarose gel electrophoresis, purified by GENECLEAN™ and ligated into pKSSINBV at the Xho I and Xba I sites. This Sindbis-HBc vector is designated pKSSIN-HBc.

Construction of a Sindbis vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Xba I to release a cDNA fragment encoding HBV-X sequences. The fragment is isolated by agarose gel electrophoresis, purified using GENECLEAN™, and inserted into pKSSINBV, pre-treated with Xho I and Xba I enzymes. This Sindbis-HBx vector is designated pKSSIN-HBx.

The above Sindbis HBV expressing vectors may also be modified to coexpress a selectable drug resistance marker dependent on the requirements of the experiment or treatment of the vector infected cells. In particular, any of the above Sindbis HBV expression vectors described may also be designed to coexpress G418 resistance. This is accomplished by incorporating an internal ribosomal entry site (Example 5) followed by the bacterial neomycin phosphotransferase gene placed 3' of the HBV coding sequences and 5' of the terminal 3' end of the vector using the multiple cloning site of the vector. These G418 resistant vector constructs can be used for selecting vector infected cells for the generation of HBV specific CTL targets in the following sections.

### D. EXPRESSION IN INFECTED CELLS WITH SINDBIS VECTORS

#### 1. ELISA

Cell lysates from cells infected by any of the HBV expressing vectors are made by washing 1.0x10<sup>7</sup> cultured cells with PBS, resuspending the cells to a total volume of 600  $\mu$ l in PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350 (Fisher, Pittsburgh, Pa.) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

Core antigen and precore antigen in cell lysates and secreted e antigen in culture supernatant are assayed using the Abbott HBe, rDNA EIA kit (Abbott Laboratories Diagnostic Division, Chicago, Ill.). Another sensitive EIA assay for precore antigen in cell lysates and secreted e antigen in culture supernatant is performed using the Incstar ETI-EB kit (Incstar Corporation, Stillwater, Minn.). A standard curve is generated from dilutions of recombinant hepatitis B core and e antigen obtained from Biogen (Geneva, Switzerland).

As shown in FIG. 16, using these procedures approximately 100-200 ng/ml HBV e antigen is expressed in the cell lysates and 300-400 ng/ml HBV e antigen is secreted from BHK cells infected with the Sin BV HB e vector.

As shown in FIG. 17, using these procedures, approximately 40 ng/ml HBV core antigen is expressed in the cell lysates from 10<sup>6</sup> BHK cells infected with the Sin BV

HBcore. Mouse fibroblast cells infected with the recombinant HBcore Sindbis vector express 6–7 fold higher HBV core protein levels than the recombinant HBcore retroviral vector transduced cells (WO 93/15207). As shown in FIG. 18, using these procedures, approximately 12–14 ng/ml HBV core antigen is expressed in the cell lysates from  $10^6$  L-M(TK-) cells infected with the SinBVHBcore vector as compared to the approximately 2 ng/ml HBV core antigen expressed from recombinant HBcore retroviral vector transducer cells.

## 2. IMMUNOPRECIPITATION/WESTERN BLOT

Characterization of the precore/core and e antigens expressed by vector infected cells is performed by immunoprecipitation followed by Western blot analysis. Specifically, 0.5–1.0 ml of cell lysate in PBS or culture supernatant is mixed with polyclonal rabbit anti-hepatitis B core antigen (DAKO Corporation, Carpinteria, Calif.) bound to protein G-Sepharose (Pharmacia LKB, Uppsala, Sweden) and incubated overnight at 4° C. Samples are washed twice in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA and boiled in sample loading buffer with 0.5% 2-mercaptoethanol. Proteins are first resolved by SDS polyacrylamide gel electrophoresis, and then transferred to Immobilon (Millipore Corp., Bedford, Me.) and probed with the DAKO polyclonal rabbit anti-hepatitis B core antigen, followed by  $^{125}$ I-protein A.

## E. TESTING IMMUNE RESPONSE

### 1. CYTOTOXICITY ASSAYS

#### (a) Inbred Mice

Six- to eight-week-old female C3H/He mice (Charles River, Ma.) are injected twice intraperitoneally (i.p.) at 1 week intervals with  $1 \times 10^6$  of Sindbis HBe or HBcore vector. Animals are sacrificed 7 or 14 days later and the splenocytes ( $3 \times 10^6$ /ml) cultured in vitro with their respective irradiated (10,000 rads) retroviral vector transduced cells ( $6 \times 10^4$ /ml) (WO 93/15207) in T-25 flasks (Corning, Corning, N.Y.). Culture medium consists of RPMI 1640, 5% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 50  $\mu$ g/ml gentamycin and  $10^{-5}$ M 2-mercaptoethanol (Sigma, St. Louis, Mo.). Effector cells are harvested 4–7 days later and tested using various effector:target cell ratios in 96 well microtiter plates (Corning, Corning, N.Y.) in a standard chromium release assay. Targets are the retroviral vector transduced L-M(TK-) cells (ATCC No. CCL 1.3) whereas the non-transduced syngeneic cell lines are used as negative controls. CTL targets may also be generated by infecting syngeneic cells with the Sindbis HBe or HBcore vector coexpressing the G418 resistance marker. Infected cells are then selected using 800 g/ml G418 for two weeks. Specifically,  $\text{Na}_2^{51}\text{CrO}_4$ -labeled (Amersham, Arlington Heights, Ill.) (100  $\mu$ Ci, 1 hour at 37° C.) target cells ( $1 \times 10^4$  cells/well) are mixed with effector cells at various effector to target cell ratios in a final volume of 200  $\mu$ l. Following incubation, 100  $\mu$ l of culture medium is removed and analyzed in a Beckman gamma spectrometer (Beckman, Dallas, Tex.). Spontaneous release (SR) is determined as CPM from targets plus medium and maximum release (MR) is determined as CPM from targets plus 1M HCl. Percent target cell lysis is calculated as:  $[(\text{Effector cell} + \text{target CPM}) - (\text{SR})] / (\text{MR}) - (\text{SR}) \times 100$ . Spontaneous release values of targets are typically 10%–20% of the MR.

For certain CTL assays, the effectors may be in vitro stimulated multiple times, for example, on day 8–12 after the primary in vitro stimulation. More specifically,  $10^7$  effector cells are mixed with  $6 \times 10^5$  irradiated (10,000 rads) stimulator cells, and  $2 \times 10^7$  irradiated (3,000 rads) “filler” cells (prepared as described below) in 10 ml of “complete”

RPMI medium. (RPMI containing: 5% heat inactivated Fetal Bovine Serum, two mM L-glutamine, 1 mM sodium pyruvate,  $1 \times$  non essential amino acids, and  $5 \times 10^{-5}$ M 2-mercaptoethanol). Stimulator cells for in vitro stimulation of effector cells are generated from irradiated retroviral vector transduced (10,000 rads) L-M (TK-) cells. “Filler” cells are prepared from naive syngeneic mouse spleen cells resuspended in RPMI, irradiated with 3,000 rads at room temperature. Splenocytes are washed with RPMI, centrifuged at 3,000 rpm for 5 minutes at room temperature, and the pellet is resuspended in RPMI. The resuspended cells are treated with 1.0 ml tris-ammonium chloride (100 ml of 0.17M tris base, pH 7.65, plus 900 ml of 0.155M  $\text{NH}_4\text{Cl}$ ; final solution is adjusted to a pH of 7.2) at 37° C. for 3–5 minutes. The secondary in vitro restimulation is then cultured for 5–7 days before testing in a CTL assay. Any subsequent restimulations are cultured as described above with the addition of 2–10 U of recombinant human IL-2 (200 U/ml, catalog #799068, Boehringer Mannheim, W. Germany).

Using these procedures, it can be shown that CTLs to HBV e antigen can be induced.

#### (b) HLA A2.1 Transgenic Mice

Six- to eight-week-old female HLA A2.1 transgenic mice (V. Engelhard, Charlottesville, Va.) are injected twice intraperitoneally (i.p.) at one week intervals with  $1.0 \times 10^6$  pfu of Sindbis vector expressing HBe or HBcore. Animals are sacrificed 7 days later and the splenocytes ( $3 \times 10^6$ /ml) cultured in vitro with irradiated (10,000 rads) retroviral vector transduced Jurkat A2/K<sup>b</sup> cells (WO 93/15207), or with peptide coated Jurkat A2/K<sup>b</sup> cells ( $6 \times 10^4$ /ml) in flasks (T-25, Corning, Corning, N.Y.). The remainder of the chromium release assay is performed as described in Example 13E 1.a, where the targets are transduced and non-transduced EL4 A2/K<sup>b</sup> (WO 93/15207) and Jurkat A2/K<sup>b</sup> cells. Non-transduced cell lines are utilized as negative controls. The targets may also be peptide coated EL4 A2/K<sup>b</sup> cells.

#### (c) Transduction of Human Cells With Vector Construct

Lymphoblastoid cell lines (LCL) are established for each patient by infecting (transforming) their B-cells with fresh Epstein-Barr virus (EBV) taken from the supernatant of a 3-week-old culture of B95-8, EBV transformed marmoset leukocytes (ATCC CRL 1612). Three weeks after EBV-transformation, the LCL are infected with Sindbis vector expressing HBV core or e antigen and G418 resistance. Vector infection of LCL is accomplished by infecting LCL cells with packaged alphavirus vector particles produced from the appropriate cell line. The culture medium consists of RPMI 1640, 20% heat inactivated fetal bovine serum (Hyclone, Logan, Utah), 5.0 mM sodium pyruvate and 5.0 mM non-essential amino acids. Infected LCL cells are selected by adding 800  $\mu$ g/ml G418. The Jurkat A<sub>2</sub>/K<sup>b</sup> cells (L. Sherman, Scripps Institute, San Diego, Calif.) are infected essentially as described for the infection of LCL cells.

#### (d) Human CTL assays

Human PBMC are separated by Ficoll (Sigma, St. Louis, Mo.) gradient centrifugation. Specifically, cells are centrifuged at 3,000 rpm at room temperature for 5 minutes. The PBMCs are restimulated in vitro with their autologous retroviral vector transduced (WO 93/15207) LCL or HLA-matched cells at an effector:target ratio of 10:1 for 10 days. Culture medium consists of RPMI 1640 with prescreened lots of 5% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate and 50  $\mu$ g/ml gentamycin. The resulting stimulated CTL effectors are tested for CTL activity using Sindbis vector infected autologous LCL or HLA-matched



cells as targets in the standard chromium release assay, Example 13 1.a. Since most patients have immunity to EBV, the non-transduced EBV-transformed B-cells (LCL) used as negative controls, will also be recognized as targets by EBV-specific CTL along with the transduced LCL. In order to reduce the high background due to killing of labeled target cells by EBV-specific CTL, it is necessary to add unlabeled non-transduced LCL to labeled target cells at a ratio of 50:1.

## 2. DETECTION OF HUMORAL IMMUNE RESPONSE

Humoral immune responses in mice specific for HBV core and e antigens are detected by ELISA. The ELISA protocol utilizes 100 µg/well of recombinant HBV core and recombinant HBV e antigen (Biogen, Geneva, Switzerland) to coat 96-well plates. Sera from mice immunized with vector expressing HBV core or HBV e antigen are then serially diluted in the antigen-coated wells and incubated for 1 to 2 hours at room temperature. After incubation, a mixture of rabbit anti-mouse IgG1, IgG2a, IgG2b, and IgG3 with equivalent titers is added to the wells. Horseradish peroxidase ("HRP")-conjugated goat anti-rabbit anti-serum is added to each well and the samples are incubated for 1 to 2 hours at room temperature. After incubation, reactivity is visualized by adding the appropriate substrate. Color will develop in wells that contain IgG antibodies specific for HBV core or HBV e antigen.

## 3. T CELL PROLIFERATION

Antigen induced T-helper activity resulting from two or three injections of Sindbis vector expressing HBV core or e antigen, is measured in vitro. Specifically, splenocytes from immunized mice are restimulated in vitro at a predetermined ratio with cells expressing HBV core or e antigen or with cells not expressing HBV core or e antigen as a negative control. After five days at 37° C. and 5% CO<sub>2</sub> in RPMI 1640 culture medium containing 5% FBS, 1.0 mM sodium pyruvate and 10<sup>-5</sup> 2-mercaptoethanol, the supernatant is tested for IL-2 activity. IL-2 is secreted specifically by T-helper cells stimulated by HBV core or e antigen, and its activity is measured using the CTL clone, CTLL-2 (ATCC TIB 214). Briefly, the CTLL-2 clone is dependent on IL-2 for growth and will not proliferate in the absence of IL-2. CTLL-2 cells are added to serial dilutions of supernatant test samples in a 96-well plate and incubated at 37° C. and 5% CO<sub>2</sub> for 3 days. Subsequently, 0.5 µCi <sup>3</sup>H-thymidine is added to the CTLL-2 cells. 0.5Ci <sup>3</sup>H-thymidine is incorporated only if the CTLL-2 cells proliferate. After an overnight incubation, cells are harvested using a PHD cell harvester (Cambridge Technology Inc., Watertown, Mass.) and counted in a Beckman beta counter. The amount of IL-2 in a sample is determined from a standard curve generated from a recombinant IL-2 standard obtained from Boehringer Mannheim (Indianapolis, Ind.).

## F. ADMINISTRATION PROTOCOLS

### 1. MICE

#### (a) Direct Vector Administration

The mouse system may also be used to evaluate the induction of humoral and cell-mediated immune responses with direct administration of Sindbis vector encoding HBV core or e antigen. Briefly, six- to eight-week-old female C3H/He mice are injected intramuscularly (i.m.) with 0.1 ml of reconstituted (with sterile deionized, distilled water) or intraperitoneally (ip) with 1.0 ml of lyophilized HBV core or HBV e expressing Sindbis vector. Two injections are given one week apart. Seven days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a.

### 2. CHIMPANZEE ADMINISTRATION PROTOCOL

The data generated in the mouse system described above is used to determine the protocol of administration of vector in chimpanzees chronically infected with hepatitis B virus. Based on the induction of HBV-specific CTLs in mice, the

subjects in chimpanzee trials will receive four doses of vector encoding core or e antigen at 7 day intervals given in two successively escalating dosage groups. Control subjects will receive a placebo comprised of formulation media. The dosage will be either 10<sup>7</sup> or 10<sup>8</sup> pfu given in four 1.0 ml injections i.m. on each injection day. Blood samples will be drawn on days 0, 14, 28, 42, 56, 70, and 84 in order to measure serum alanine aminotransferase (ALT) levels, the presence of hepatitis B e antigen, the presence of antibodies directed against the hepatitis B e antigen, serum HBV DNA levels and to assess safety and tolerability of the treatment. The hepatitis B e antigen and antibodies to HB e antigen is detected by Abbott HB e rDNA ELA kit (Abbott Laboratories Diagnostic Division, Chicago, Ill.) and the serum HBV DNA levels is determined by the Chiron bDNA assay. Efficacy of the induction of CTLs against hepatitis B core or e antigen can be determined as in Example 13E 1.c.

Based on the safety and efficacy results from the chimpanzee studies, the dosage and inoculation schedule is determined for administration of the vector to subjects in human trials. These subjects are monitored for serum ALT levels, presence of HBV e antigen, the presence of antibodies directed against the HBV e antigen and serum HBV DNA levels essentially as described above. Induction of human CTLs against hepatitis B core or e antigen is determined as in Example 13E 1.c.

## G. GENERATION OF ELVIS VECTOR CONSTRUCTS WHICH EXPRESS HBV ANTIGENS FOR THE INDUCTION OF AN IMMUNE RESPONSE

### 1. CONSTRUCTION OF ELVIS VECTORS EXPRESSING HBVE-C, HBV CORE AND HBV X

Construction of an ELVIS vector expressing the HBV e antigen is accomplished by digesting the SK<sup>+</sup>HB e-c plasmid with Xho I and Not I to release the cDNA fragment encoding HBVe-c sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN<sup>™</sup>, and inserted into pVGELVIS-SINBV-linker vector, previously prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBe.

The HBcore PCR product described previously is digested with Xho I and Cla I, isolated by agarose gel electrophoresis, purified using GENECLEAN<sup>™</sup>, and ligated into SK<sup>+</sup>II (Bluescript, Stratagene, Calif.) digested with Xho I and Cla I. This construct is designated SK<sup>+</sup>HBcore. Construction of the ELVIS vector expressing the HBV core sequence is accomplished by digesting the SK<sup>+</sup>HBcore plasmid with Xho I and Not I to release the cDNA fragment encoding HBVcore sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN<sup>™</sup>, and inserted into pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBcore.

Construction of the ELVIS vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-XAg with Xho I and Not I to release the cDNA fragment encoding HBV-X sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN<sup>™</sup>, and inserted into the pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBX.

Any of the above three constructs can be used for selecting vector infected cells for the generation of HBV specific CTL targets in the following sections.

### 2. EXPRESSION OF TRANSFECTED CELLS WITH ELVIS VECTORS

The pVGELVIS-HBe plasmid DNA is isolated and purified, and 2 µg of pVGELVIS-HBe DNA is complexed with 10 µl of LIPOFECTAMINE<sup>™</sup> and transfected into 2x10<sup>5</sup> BHK cells contained in 35 mM petri plates. Two days post-transfection, supernatants and whole cell lysates were



collected and an ELISA assay (see below) was used to determine the amount of expressed HBV-e antigen.

Cell lysates from cells infected by any of the sibling pVGELVIS-HBe vectors transfected, are made by washing  $1.0 \times 10^6$  cultured cells with PBS, resuspending the cells to a total volume of 600  $\mu$ l in PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350 (Fisher, Pittsburgh, Pa.) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

Core antigen and precore antigen in cell lysates and secreted e antigen in culture supernatant are assayed using the Abbott HBe, rDNA EIA kit (Abbott Laboratories Diagnostic Division, Chicago, Ill.). Another sensitive EIA assay for precore antigen in cell lysates and secreted e antigen in culture supernatant is performed using the Incstar ETI-EB kit (Incstar Corporation, Stillwater, Minn.). A standard curve is generated from dilutions of recombinant hepatitis B core and e antigen obtained from Biogen (Geneva, Switzerland).

As shown in FIG. 19, using these procedures, approximately 2 ng/ml HBV e antigen is expressed in the cell lysates and also secreted from BHK cells transfected with different clones of the pVGELVIS-HBe plasmid.

Characterization of the precore/core and e antigens expressed by vector transfected cells is performed by immunoprecipitation followed by Western blot analysis. Specifically, 0.5–1.0 ml of cell lysate in PBS or culture supernatant is mixed with polyclonal rabbit anti-hepatitis B core antigen (DAKO Corporation, Carpinteria, Calif.) bound to protein G-Sepharose (Pharmacia LKB, Uppsala, Sweden) and incubated overnight at 4° C. Samples are washed twice in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA and boiled in sample loading buffer with 0.5% 2-mercaptoethanol. Proteins are first resolved by SDS polyacrylamide gel electrophoresis, and then transferred to Immobilon (Millipore Corp., Bedford, Me.) and probed with the DAKO polyclonal rabbit anti-hepatitis core B antigen, followed by  $^{125}$ I-protein A.

### 3. TESTING IMMUNE RESPONSE

#### (a) Administration Protocols

The mouse model system is also used to evaluate the induction of humoral and cell-mediated immune responses following direct administration of ELVIS vector expressing HBV core or e antigen. Briefly, six- to eight-week-old female Balb/c, C57B1/6, C3H/He mice (Charles River, Mass.) and HLA A2.1 transgenic mice (V. Engelhard, Charlottesville, Va.) are injected intramuscularly (i.m.) with, for example, 50  $\mu$ g or greater, pVGELVIS-HBcore, pVGELVIS-HBe or pVGELVIS-HBX vector DNA. Two injections are given one week apart. Seven or fourteen days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a. Detection of humoral immune responses in mice is performed essentially as described in Example 13E 2 and detection of T cell proliferation in mice is performed essentially as described in Example 13E 3.

#### Example 14

#### SINDBIS VECTORS EXPRESSING VIRAL PROTEINS FOR INDUCTION OF THE IMMUNE RESPONSE OR FOR BLOCKING VIRUS HOST CELL INTERACTIONS

The following example describes procedures for constructing Sindbis vectors capable of generating an immune response by expressing an HIV viral antigen. Methods are also given to test expression and induction of an immune response.

#### SINDBIS VECTORS USED TO ELICIT AN IMMUNE RESPONSE

##### A. HIV IIIB ENV EXPRESSION VECTOR

A 2.7 Kb Kpn I-Xho I DNA fragment was isolated from the HIV proviral clone BH10-R3 (for sequence, see Ratner et al., *Nature* 313:277, 1985) and a ~400 bp Sal I-Kpn I DNA fragment from IIIexE7deltaenv (a Bal31 deletion to nt 5496) was ligated into the Sal I site in the plasmid SK<sup>+</sup>. From this clone, a 3.1 kb env DNA fragment (Xho I-Not I) was purified and ligated into the previously described Sindbis vectors predigested with Xho I and NotI.

##### B. CREATION OF A PRODUCER CELL LINE WHICH EXPRESSES HIV SPECIFIC ANTIGENS

To construct a vector producing cell line that expresses the HIV IIIB env derived from the vector described above, in vitro transcribed RNA transcripts are transfected in a Sindbis packaging cell line (Example 7). Specifically, the Sindbis RNA vector molecules are initially produced by using a SP6 in vitro transcribed RNA polymerase system used to transcribe from a cDNA Sindbis vector clone encoding the HIV specific sequences. The generated in vitro RNA vector products, are then transfected into a Sindbis packaging or hopping cell line which leads to the transient production of infectious vector particles within 24 hours. These vector particles are then collected from the supernatants of the cell line cultures and then filtered through a 0.45 micron filter to avoid cellular contamination. The filtered supernatants are then used to infect a fresh monolayer of Sindbis packaging cells. Within 24 hours of infection, Sindbis vector particles are produced containing positive stranded Sindbis recombinant RNA encoding Sindbis non-structural proteins and HIV specific sequences.

An alternative configuration of a Sindbis HIV IIIB env vector is a promoter driven cDNA Sindbis construct containing a selectable marker. In this configuration the above-described Xho I to NotI fragment containing the specific HIV IIIB env sequence is placed in a similar cDNA Sindbis vector driven by a constitutive promoter in place of a bacteriophage polymerase recognition sequence. Using this configuration, the expression vector plasmids are transfected into the packaging cell line and selected for the drug resistance gene 24 to 48 hour post-transfection. Resistant colonies are then pooled 14 days later (dependent on the selection marker used) and diluted cloned. Several dilution clones are then propagated, and assayed for highest vector titer. The highest titer clones are then expanded and stored frozen. The stored clones are tested for HIV specific protein production and immune response induction.

##### C. TESTING FOR HIV SPECIFIC PROTEIN PRODUCTION AND AN IMMUNE RESPONSE

Cell lysates from the Sindbis HIV producer cell line are tested for HIV specific protein production by Western blot analysis. To test the ability of the vector to transfer expression in vitro, BHK-21 cells are infected with filtered supernatant containing viral vector and assayed by Western blot analysis 24 hours post infection. Once protein expression has been verified in vivo mouse and primate studies can be performed to demonstrate the ability of syngeneic cells expressing a foreign antigen after vector treatment to: (a) elicit a CTL response in mice by injecting either infected syngeneic cells or preparations of infectious vector; (b) elicit CTL responses in a human in vitro culture system; (c) to infect human, chimpanzee and macaque cells, including primary cells, so that these can be used to elicit CTL responses and can serve as targets in CTL assays; (d) map immune response epitopes; and (e) elicit and measure CTL responses to other non-HIV antigens such as mouse CMV (MCMV).

# 1. IMMUNE RESPONSE TO SINDBIS VIRAL VECTOR-ENCODED ANTIGENS

To test the immune response elicited from a cell line transduced with a Sindbis HIV IIIB env vector, a murine tumor cell line (B/C10ME) (H-2<sup>d</sup>) (Patek et al., *Cell. Immunol.* 72:113, 1982) is infected with a recombinant Sindbis virus carrying the HIV IIIB vector. The HIV env expressing cell line (B/C10ME-IIIB) was then utilized to stimulate HIV env-specific CTL in syngeneic (i.e., MHC identical) Balb/c (H-2<sup>d</sup>) mice. Mice are immunized by intraperitoneal injection with B/C10ME-IIIB cells (1×10<sup>7</sup> cells) and boosted on day 7–14. (Boosting may not be required.) Responder spleen cell suspensions are prepared from these immunized mice and the cells cultured in vitro for 4 days in the presence of either B/C10ME-IIIB (BCenv) or B/C10ME (BC) mitomycin-C-treated cells at a stimulator:responder cell ratio of 1:50. The effector cells are harvested from these cultures, counted, and mixed with radiolabeled (<sup>51</sup>Cr) target cells (i.e., B/C10MEenv-29 or B/C10ME) at various effector:target (E:T) cell ratios in a standard 4–5 hour <sup>51</sup>Cr-release assay. Following incubation, the microtitre plates are centrifuged, 100 μl culture supernate is removed, and the amount of radiolabel released from lysed cells quantitated in a Beckman gamma spectrometer. Target cell lysis was calculated as: % Target Lysis = (Exp CPM – SR CPM) / (MR CPM – SR CPM) × 100, where experimental counts per minute (Exp CPM) represents effectors plus targets; spontaneous release (SR) CPM represents targets alone; and maximum release (MR) CPM represents targets in the presence of 1M HCl.

# 2. STIMULATION OF AN IMMUNE RESPONSE IN MICE BY DIRECT INJECTION OF RECOMBINANT SINDBIS VECTOR

Experiments are performed to evaluate the ability of recombinant Sindbis viral vectors to induce expression of HIV envelope proteins following direct injection in mice. Approximately 10<sup>4</sup> to 10<sup>5</sup> (pfu) of recombinant Sindbis virus carrying the HIV IIIB env vector construct are injected twice (2x) at 3-week intervals either by the intraperitoneal (i.p.) or intramuscular (i.m.) route. This amount of Sindbis virus is determined to be less than the amount considered to

This blocking action may occur intracellularly, on the cell membrane, or extracellularly. The blocking action of a viral or, in particular, a Sindbis vector carrying a gene for a blocking agent, can be mediated either from inside a susceptible cell or by secreting a version of the blocking protein to locally block the pathogenic interaction.

In the case of HIV, the two agents of interaction are the gp 120/gp 41 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker would be a vector construct expressing either an HIV env analogue that blocks HIV entry without causing pathogenic effects, or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp 41 is secreted or produced only intracellularly so as to protect only the vector-containing cell. It may be advantageous to add human immunoglobulin heavy chains or other components to CD4 in order to enhance stability or complement lysis. Delivery of a Sindbis vector encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule.

Vector particles leading to expression of HIV env analogues may also be constructed as described above. It will be evident to one skilled in the art which portions are capable of blocking virus adsorption without overt pathogenic side effects (Willey et al., *J. Virol.* 62:139, 1988; Fisher et al., *Science* 233:655, 1986).

## Example 15

### A. CONSTRUCTION OF FIV ENV/REV/RRE SINDBIS VECTOR FOR THE INDUCTION OF AN IMMUNE RESPONSE

Sequences encoding the FIV env/rev/RRE gene are amplified and isolated from plasmid pFIV-14-Petaluma (NIH Research and Reference Reagent Program, Maryland) using the following primers:

The sense primer sequence has two consecutive Xho I restriction sites that are placed at the 5' end at position 6020 of clone 34F10 (Talbot et al., *PNAS* 86:5743–5747, 1989): (SEQ.ID NO. 105)

---

5'-3': CC CTC GAG CTC GAG GGG TCA CTG AGA AAC TAG AAA AAG AAT TAG

---

stimulate an immune response. Spleen cells are prepared for CTL approximately 7 to 14 days after the second injection of vector.

The antisense primer sequence is complementary to a sequence at position 9387 of clone 34F10. The 5' end of the primer has a Not I site (SEQ.ID NO. 106)

---

5'-3': CC GCG GCC GC GTA TCT GTG GGA GCC TCA AGG GAG AAC

---

### D. BLOCKING AGENTS DERIVED FROM VIRAL PROTEIN ANALOGUES EXPRESSED FROM RECOMBINANT SINDBIS VECTORS

Many infectious diseases, cancers, autoimmune diseases, and other diseases involve the interaction of viral particles with cells, cells with cells, or cells with factors. In viral infections, viruses commonly enter cells via receptors on the surface of susceptible cells. In cancers, cells may respond inappropriately or not at all to signals from other cells or factors. In autoimmune disease, there is inappropriate recognition of "self" markers. These interactions may be blocked by producing an analogue to either of the partners in an interaction, in vivo.

55

The PCR product is then placed in the pBluescript KSII+ plasmid (Stratagene, Calif.) and verified by DNA sequencing. This construct is designated pBluescript KSII+ FIV env/rev/RRE. The Xho I-Not I fragment is then excised and inserted into the Sindbis backbone.

60

Construction of a Sindbis vector expressing the FIV env/rev/RRE sequence is accomplished by digesting the SK+ FIV env/rev/RRE plasmid with Xho I and Not I restriction enzyme sites to release the cDNA fragment encoding FIV env/rev/RRE sequences. The fragment is then isolated by agarose gel electrophoresis, purified by GENECLAN™ and inserted into the desired Sindbis vector backbone,

65

prepared by digestion with Xho I and Not I. The Sindbis vectors described in Example 3, are suitable for the insertion of the FIV env/rev/RRE sequences. Such Sindbis vectors include pKSSINBV, pKSSINd1JRsrc, pKSSINd1JRsjrPC, pKSSINd1JRsjrNP(7582-7601) and pKSSINd1JRsexjr.

The above Sindbis FIV env/rev/RRE expressing vectors may also be modified to coexpress a selectable drug resistance marker dependent on the requirements of the experiment or treatment of the vector infected cells. Any of the above Sindbis FIV env/rev/RRE expression vectors described may also be designed to coexpress for G418 resistance. This is accomplished by incorporating an internal ribosomal entry site (Example 5) followed by the bacterial neomycin phosphotransferase gene placed 3' of the FIV env/rev/RRE coding sequences and 5' of the terminal 3' end of the vector using the multiple cloning site of the vector. These G418 resistant vector constructs can be used for selecting vector infected cells for the generation of FIV env/rev/RRE specific CTL targets in the following sections.

#### B. INFECTION OF FELINE CELLS WITH SINDBIS VECTOR EXPRESSING FIV ENV/REV/RRE

The feline kidney cell line (CRFK) is grown in DMEM containing 10% FBS. CRFK cells are infected with the Sindbis vector as described in Examples 3 and 7, and used to show vector expression in feline cells using Western blot analysis.

#### C. EXPRESSION OF INFECTED CELLS

Cell lysates from cells infected by any of the FIV env/rev/RRE expressing vectors are made by washing  $1.0 \times 10^7$  cultured cells with PBS, resuspending the cells to a total volume of 600  $\mu$ l in PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350 (Fisher, Pittsburgh, Pa.) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

Proteins are separated according to their molecular weight (MW) by means of SDS polyacrylamide gel electrophoresis. Proteins are then transferred from the gel to a IPVH Immobilon-P membrane (Millipore Corp., Bedford, Mass.). The Hoefer HSI TTE transfer apparatus (Hoefer Scientific Instruments, Calif.) is used to transfer proteins from the gel to the membrane. The membrane is then probed with either CE4-13B1 or CE3-81, monoclonal antibodies directed against FIV env gp100. The bound antibody is detected using  $^{125}$ I-labeled protein A, which allows visualization of the transduced protein by autoradiography.

#### D. TESTING CELLULAR IMMUNE RESPONSE

##### 1. INBRED MICE

Six- to eight-week-old female Balb/c (H-2d), C57B 1/6 (H-2b) and C3H/He (H-2k) mice (Charles River, Mass.) are injected twice intraperitoneally (i.p.) at 1 week intervals with  $1 \times 10^6$  pfu of Sindbis FIV env/rev/RRE vector. Animals are sacrificed 7 days later and the splenocytes ( $3 \times 10^6$ /ml) cultured in vitro with their respective irradiated (10,000 rads) retroviral vector transduced syngeneic cells (WO 94/06921) ( $6 \times 10^4$ /ml) in T-25 flasks (Corning, Corning, N.Y.). These transduced cells include the murine fibroblast cell lines BC10ME (H-2d) (ATCC No. TIB85), B16 (H-2b) and L-M(TK-) (H-2k) (ATCC No. CCL 1.3). These cell lines are grown in DMEM containing 4500 mg/L glucose, 584 mg/L L-glutamine (Irvine Scientific, Santa Ana, Calif.) and 10% FBS (Gemini, Calabasas, Calif.). Culture medium consists of RPMI 1640, 5% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 50 g/ml gentamycin and  $10^{-5}$ M 2-mercaptoethanol (Sigma, St. Louis, Mo.). Effector cells are harvested 4-7 days later and tested using various effector:target cell ratios in 96 well microtiter plates

(Corning, Corning, N.Y.) in a standard chromium release assay. Targets are the retroviral vector transduced syngeneic cells (WO 94/06921) whereas the non-transduced syngeneic cell lines are used as negative controls. CTL targets may also be generated from infecting syngeneic cells with the Sindbis FIV env/rev/RRE vector coexpressing the G418 resistance marker. Infected cells are then selected using 800  $\mu$ g/ml G418 for two weeks. Specifically,  $\text{Na}_2^{51}\text{CrO}_4$ -labeled (Amersham, Arlington Heights, Ill.) ( $100 \mu\text{Ci}$ , 1 hour at  $37^\circ \text{C}$ .) target cells ( $1 \times 10^4$  cells/well) are mixed with effector cells at various effector to target cell ratios in a final volume of 200  $\mu$ l. Following incubation, 100  $\mu$ l of culture medium is removed and analyzed in a Beckman gamma spectrometer (Beckman, Dallas, Tex.). Spontaneous release (SR) is determined as CPM from targets plus medium and maximum release (MR) is determined as CPM from targets plus 1M HCl. Percent target cell lysis is calculated as:  $[(\text{Effector cell} + \text{target CPM}) - (\text{SR})] / [(\text{MR}) - (\text{SR})] \times 100$ . Spontaneous release values of targets are typically 10%-20% of the MR.

For certain CTL assays, the effectors may be in vitro stimulated multiple times, for example, on day 8-12 after the primary in vitro stimulation. More specifically,  $10^7$  effector cells are mixed with  $6 \times 10^5$  irradiated (10,000 rads) stimulator cells, and  $2 \times 10^7$  irradiated (3,000 rads) "filler" cells (prepared as described below) in 10 ml of "complete" RPMI medium. (RPMI containing: 5% heat inactivated Fetal Bovine Serum, 2 mM L-glutamine, 1 mM sodium pyruvate,  $1 \times$  non essential amino acids, and  $5 \times 10^{-5}$ M 2-mercaptoethanol). Stimulator cells for in vitro stimulation of effector cells are generated from irradiated retroviral vector transduced syngeneic cells. "Filler" cells are prepared from naive syngeneic mouse spleen cells resuspended in RPMI, irradiated with 3,000 rads at room temperature. Splenocytes are washed with RPMI, centrifuged at 3,000 rpm for 5 minutes at room temperature, and the pellet is resuspended in RPMI. The resuspended cells are treated with 1.0 ml tris-ammonium chloride (100 ml of 0.17 M tris base, pH 7.65, plus 900 ml of 0.155M  $\text{NH}_4\text{Cl}$ ; final solution is adjusted to a pH of 7.2) at  $37^\circ \text{C}$ . for 3-5 minutes. The secondary in vitro restimulation is then cultured for 5-7 days before testing in a CTL assay. Any subsequent restimulations are cultured as described above with the addition of 2-10 U of recombinant human IL-2 (200  $\mu$ g/ml, catalog #799068, Boehringer Mannheim, W. Germany).

##### 2. FELINES

Since the vectors are to be utilized for treating felines, an assay demonstrating immunological efficacy in felines is needed. The following is a description of the generation of the autologous T-cell lines needed for restimulator and target cells for the standard  $^{51}\text{Cr}$  release assay (Brown et al., *J. Vir.* 65:3359-3364, 1991). Briefly, peripheral blood mononuclear cells (PBMC) are obtained following venipuncture and Ficoll-sodium diatrizoate (Histopaque-1077; Sigma, St. Louis, Mo.) density gradient centrifugation. These PBMCs are stimulated by 5  $\mu$ g/ml concanavalin A (Con A, Sigma) for three days, and maintenance in medium containing 25 u/ml human recombinant interleukin-2 (IL-2) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and 10% bovine T-cell growth factor (TCGF). Cells are seeded into round bottom 96-well microtiter plates at an average of 1 or 0.3 cells per well with  $5 \times 10^4$  irradiated (3,000 rads) autologous PBMC, 10% bovine TCGF, and 25 u/ml of IL-2 in a final volume of 200  $\mu$ l of complete RPMI. Complete RPMI consist of RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine,  $5 \times 10^{-5}$  M 2-mercaptoethanol, and 50  $\mu$ g of gentamycin per ml. Clones are expanded sequentially to 48-well and 24-well plates. After several weeks, cells are

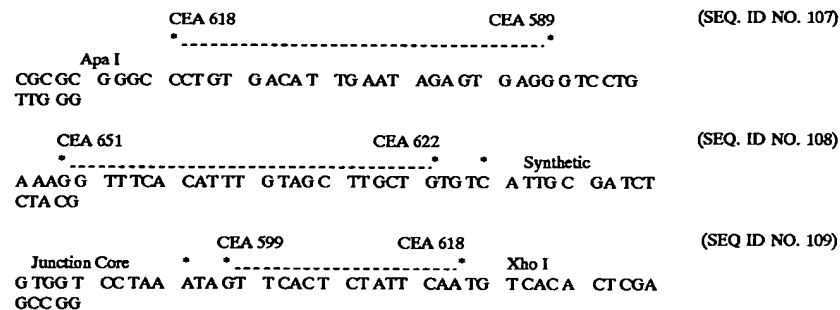
transduced with retroviral vectors expressing FIV env/rev genes (WO 94/06921), and selected with G418. Expression of these cell lines are monitored by Western blot analysis as in Example 15C. Cell lines expressing high levels of the desired protein function as stimulators and targets in a standard  $^{51}\text{Cr}$  release assay as in Example 15 D 1. Effector cells are recovered for the CTL assay from the peripheral blood mononuclear cells (PBMC) obtained following venipuncture and Ficoll-sodium diatrizoate density gradient centrifugation.

#### E. ADMINISTRATION PROTOCOLS

Six- to eight-week-old female Balb/C, C57B16 or C3H/He mice are injected intramuscularly (i.m.) with 0.1 ml of reconstituted (with sterile deionized, distilled water) or intraperitoneally (i.p.) with 1.0 ml of lyophilized FIV env/rev/RRE expressing Sindbis vector. Two injections are given one week apart. Seven days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13 D 1.

Felines are also injected intramuscularly (i.m.) with 0.5 ml of reconstituted (with sterile deionized, distilled water) or intraperitoneally (i.p.) with 2.0 ml of lyophilized FIV env/rev/RRE expressing Sindbis vector. Two injections are given one week apart. Seven days after the second injection,

region. In choosing the correct A1 and B 1 antisense sequences, the only two requirements are that they be specific for the targeted RNA sequence and that the antisense sequences hybridize to two RNA sequence domains separated by three nucleotides. This three nucleotide gap will serve as a hinge domain for the polymerase to hop and switch reading strands bridging the non-structural protein domain of the vector to the junction region of the vector (FIG. 5). To construct such a configuration, two oligonucleotides are synthesized complementing each other to create a fragment insert containing convenient restriction enzyme sites at the extreme 5' and 3' ends. The oligonucleotide fragment insert is then ligated into the Sindbis vector between the disabled junction region and the multiple cloning sites of the Sindbis vector. The sense oligonucleotide strand, from 5' to 3', should contain an Apa I restriction site, followed by the A1 anti-sense domain, a six bp hinge domain, a B1 anti-sense domain, a synthetic junction region domain, and the A2 sense domain, followed by a Xho I restriction enzyme site. The following oligonucleotide sequence is used to design a CEA RNA responsive Sindbis vector. The nucleotide number sequence is obtained from Beauchemin et al., *Molec. and Cell Biol.* 7:3221, 1987. 5'-3' CEA sense strand:



PBMCs are withdrawn for the CTL assay. Chromium release CTL assays are then performed essentially as described in Example 13 D 2.

#### Example 16

##### TISSUE SPECIFIC EXPRESSION BY ACTIVATION OF DISABLED ALPHAVIRUS VECTORS USING TISSUE SPECIFIC CELLULAR RNA: CONSTRUCTION OF ALPHAVIRUS TUMOR SPECIFIC EXPRESSION VECTORS FOR THE TREATMENT OF COLORECTAL CANCER

##### A. CONSTRUCTION OF A RECOMBINANT SINDBIS VECTOR (SIN-CEA) DEPENDENT ON THE EXPRES- SION OF THE CEA TUMOR MARKER

As described previously and shown diagrammatically in FIG. 20, the disabled junction loop out model is constructed with the junction region of the vector flanked by inverted repeat sequences which are homologous to the RNA of choice. In this example, sequences from the CEA tumor antigen CDNA (Beauchemin et al., *Molec. and Cell Biol.* 7:3221, 1987) are used in the inverted repeats. To construct a CEA RNA responsive Sindbis vector, the junction region is preceded by two CEA anti-sense sequence domains (A<sup>1</sup> and B<sup>1</sup>) separated by a six base pair hinge domain. A single twenty base pair CEA sense sequence (A2), which is complementary to A1, is placed at the 3' end of the junction

40 The 5-3' CEA anti-sense strand is complementary to the above oligonucleotide. After both oligonucleotides are synthesized, the oligonucleotides are mixed together in the presence of 10 mM Mg, heated to 100° C. for 5 minutes and cooled slowly to room temperature. The oligonucleotide pair is then digested with the Apa I and Xho I restriction enzymes, mixed and ligated at a 25:1 molar ratio of insert to plasmid, pCMV-SIN or pMET-SIN predigested with the same enzymes. These constructs are designated pCMV/SIN-CEA and pMET/SIN-CEA, respectively.

##### 50 CONSTRUCTION OF A SIN-CEA VECTOR AND PRO- DUCER CELL LINE EXPRESSING GAMMA INTER- FERON (SIN-CEA/IFN)

The human gamma interferon gene is subcloned from the retroviral vector plasmid pHu-IFN- $\gamma$  (Howard et al., *Ann N.Y. Acad. Sci.* 716:167-187, 1994) by digesting with Xho I and Cla I. The resulting 500 bp fragment containing the coding sequences of  $\gamma$ -IFN is isolated from a 1% agarose gel.

Alternatively, the human  $\gamma$ -IFN cDNA is derived from RNA isolated from PHA-stimulated Jurkat T cells by guanidium thiocyanate extraction followed by ultracentrifugation through a CsCl gradient. The RNA (Sigma, St. Louis, Mo.) is then reverse-transcribed in vitro and a gene-specific oligonucleotide pair is used to amplify  $\gamma$ -IFN cDNA by polymerase chain reaction using Taq polymerase. The PCR DNA was repaired with T4 DNA polymerase and Klenow and cloned into the Hinc II site of SK+ plasmid (Stratagene, San Diego, Calif.) treated with CIAP. In the sense

orientation, the 5' end of the cDNA is adjacent to the Xho I site of the SK+ polylinker and the 3' end adjacent to the NotI site. The 512 base pair fragment encoding the human  $\gamma$ -IFN molecule is placed into the Xho I / NotI site of either the pCMV/SIN-CEA or pMET/SIN-CEA vectors. These new plasmids are designated pCMV/SIN-CEA/IFN- $\gamma$  or pMET/SIN-CEA/IFN- $\gamma$ , respectively.

#### B. CONSTRUCTION OF A SIN-CEA VECTOR AND PRODUCER CELL LINE EXPRESSING THYMIDINE KINASE (SIN-CEA/TK)

A PCR amplified product containing the cDNA clone of the herpes simplex thymidine kinase ("HSVTK"), flanked with 5' Xho I and 3' NotI restriction enzyme sites is obtained using the pHS1TK3KB (Mcknight et al., *Nuc. Acids Res.* 8:5949, 1980) clone as target DNA. The sequences for the primers used for the PCR amplification are obtained from published sequences (Wagner et al., *PNAS* 78:1442, 1981). The 1,260 base pair amplified product is then digested with Xho I and NotI ligated into the Xho I/NotI site of either the pCMV/SIN-CEA or pMET/SIN-CEA vectors. These new plasmids are designated pCMV/SIN-CEA/HSVTK or pMET/SIN-CEA/HSVTK, respectively.

#### C. CREATION OF CEA RNA DEPENDENT SINDBIS VECTOR PRODUCER CELL LINES

Unlike the previous examples of creating producer cell lines (Example 7), it may be that only a single round of gene transfer into the packaging cell line is possible by vector transfection. Since these vectors will be disabled and prevented in the synthesis of full genomic vectors, re-infection of a fresh layer of Sindbis packaging cell lines will end in an aborted infection since these vectors are now dependent on the presence of the CEA RNA to become active. Higher titers may be achieved by dilution cloning transected producer cell lines using the RT-PCR technique.

#### Example 17

##### REPLACEMENT GENE THERAPY USING RECOMBINANT ALPHAVIRUS VECTORS

The following example describes the construction of alphavirus vectors capable of generating a therapeutic protein.

#### A. CONSTRUCTION OF A SINDBIS FACTOR VIII VECTOR

Hemophilia A disease is characterized by the absence of Factor VIII, a blood plasma coagulating factor. Approximately 1 in 20,000 males have hemophilia A in which the disease state is presented as a bleeding disorder, due to the inability of affected individuals to complete the blood clotting cascade.

The treatment of individuals with hemophilia A is replacement with the Factor VIII protein. The only source for human Factor VIII is human plasma. In order to process human plasma for Factor VIII purification, human donor samples are pooled in lots of over 1000 donors. Due to the instability of the Factor VIII protein, the resulting pharmaceutical products are highly impure, with an estimated purity by weight of approximately 0.04%. In addition, there is a serious threat of such infectious diseases as hepatitis B virus and the Human Immunodeficiency Virus, among others, which contaminate the blood supply and can thus be potentially co-purified with the Factor VIII protein.

The Factor VIII cDNA clone is approximately 8,000 bps. Insertion of the Factor VIII cDNA into pKSSINBV yields a vector/heterologous gene genomic size of approximately 15,830 bps. If the packaging of this large vector RNA into particles is inefficient, the size of the insert can be decreased

further by eliminating the "B-domain" of the Factor VIII insert. It has been shown that the Factor VIII B-domain region can be removed from the cDNA without affecting the functionality of the subsequently expressed protein.

A Sindbis-Factor VIII vector is constructed as follows. Factor VIII cDNA is obtained from clone pSP64-VIII, an ATCC clone under the accession number 39812, containing a cDNA encoding the full-length human protein. pSP64-VIII is digested with Sal I, the termini are blunted with T4 DNA polymerase and 50  $\mu$ M of each dNTP, and the ca. 7700 bp. fragment is electrophoresed in a 1% agarose/TBE gel and purified with GENECLEAN™. The Factor VIII cDNA containing blunt ends is then ligated into pKSI3' SIN (Example 3), prepared by digestion with Hinc II, treated with CIAP, and purified from a 1% agarose gel. This plasmid is known as pF83' SIN.

For insertion of Factor VIII into the various Sindbis vectors described in Example 3, plasmid pF83' SIN is digested with Xho I and a limited Sac I digest, and the resulting 7,850 bp fragment is isolated from a 0.7% agarose/TBE gel. This Factor VIII-3' SIN fragment is then inserted into each of the vectors listed below. Prior to insertion of this fragment the plasmids are prepared by digestion with Xho I and Sac I, treated with CIAP, isolated by 1% agarose/TBE gel electrophoresis, and purified with GENECLEAN™:

Vector	Functional Junction Region (+/-)
pKSSINBV	+
pKSSINd1JRsjrc	+
pKSSINd1JRsjrPC	+
pKSSINd1JRsjrNP(7,582-7,601)	+
pKSSINd1JRsejxr	+

Following insertion of the Factor VIII cDNA, these vectors are designated:

pKSSINBVF8  
pKSSINd1JRsjrcF8  
pKSSINd1JRsjrPCF8  
pKSSINd1JRsjrNP(7,582-7,601)F8  
pKSSINd1JRsejxrF8  
respectively.

Packaging of the Factor VIII cDNA containing vectors is accomplished by the transfection of packaging cell lines (described in Example 7) with in vitro transcribed vector/ Factor VIII RNA. The efficiency of packaging is determined by measuring the level of Factor VIII expression in cells infected with the packaged vector and compared to similar experiments performed with the pKSSIN-luc vector described in Example 3.

#### B. CONSTRUCTION OF A GLUCOCEREBROSIDASE SINDBIS VECTOR

Gaucher disease is a genetic disorder that is characterized by the deficiency of the enzyme glucocerebrosidase. This enzyme deficiency leads to the accumulation of glucocerebroside in the lysosomes of all cells in the body. However, the disease phenotype is manifested only in the macrophages, except in the very rare neuropathic forms of the disease. The disease usually leads to enlargement of the liver and spleen and lesions in the bones. (For a review, see *Science* 256:794, 1992, and *The Metabolic Basis of Inherited Disease*, 6th ed., Scriver et al., vol. 2, p. 1677.)

A glucocerebrosidase Sindbis vector is constructed as follows. Briefly, a glucocerebrosidase (GC) cDNA clone containing a Xho I restriction enzyme site 5' and 3' of the cDNA coding sequence is first generated. The clone is generated by digesting pMFG-GC (Ohashi et al., *PNAS* 89:11332, 1992) with Nco I, blunt-ending the termini with

T4 DNA polymerase and dNTPs, ligating with Xho I linkers, and purifying the GC gene from a 1% agarose gel. The GC fragment is subsequently digested with Xho I and ligated with the desired Sindbis vector (for example, pKSSINBV) that has also been digested with Xho I. Packaging of the Sindbis-glucocerebrosidase vector is accomplished by introduction of vector RNA (for example, transfection of in vitro transcribed RNA) into any of the packaging cell lines described in Example 7.

Both the Sindbis Factor VIII and the Sindbis Glucocerebrosidase vectors are also readily convertible to plasmid DNA based-vectors which initiate vector replication and heterologous gene expression for use in direct delivery or the establishment of vector producer cell lines (see Examples 3 and 7).

#### Example 18

#### INHIBITION OF HUMAN PAPILLOMA VIRUS PATHOGENICITY BY SEQUENCE-SPECIFIC ANTISENSE OR RIBOZYME MOLECULES EXPRESSED FROM SINDBIS VIRUS VECTORS

To date, more than sixty types of human papilloma viruses (HPV), which have a pronounced tropism for cells of epithelial origin, have been isolated and characterized. Among the HPV group are a substantial number of types which infect the human anogenital tract. This group of HPVs can be further subdivided into types which are associated with benign or with malignant proliferation of the anogenital tract.

There are between 13,000 and 20,000 cervical cancer deaths per year in the U.S. In developing countries, cervical cancer is the most frequent malignancy, and in developed countries cervical cancer ranks behind breast, lung, uterus, and ovarian cancers. One statistic which especially supports the notion that anogenital proliferation is a growing health problem is that medical consultations for genital warts increased from 169,000 in 1966 to greater than 2 million in 1988.

Several lines of evidence exist which link HPV to the pathogenesis of cervical proliferative disease. A distinct

subset of types, so called 'low risk HPVs', are associated with benign proliferative states of the cervix (e.g., HPV 6,

11, 43, 44), while another subset of types, the 'high risk HPVs', are associated with lesions which may progress to the malignant state (e.g., HPV 16, 18, 31, 33, 35, etc.). Approximately 95% of cervical tumors contain HPV, with HPV type 16 or 18 DNA being found in about 70% of them.

The frequency of HPV in the young sexually active female population appears to be quite high. Indeed, in a recent study of 454 college women, 213, or 46% were HPV positive. Among the HPV positive group, 3% were HPV 6/11 positive, and 14% were HPV 16/18 positive. Of these 454 women, 33 (7.3%) had abnormal cervical proliferation, as determined by cytology.

With regard to the design of antisense and ribozyme therapeutic agents targeted to HPV, there are important

parameters to consider relating to the HPV types to target (i.e., types associated with condyloma acuminatum or types associated with malignant cervical proliferation) and HPV expressed genes to target, including but not limited to, HPV genes E2, E6, or E7.

In general, the expression of HPV genes is defined temporally in two phases, early (E) genes expressed prior to viral DNA replication, and late (L) genes expressed after viral DNA replication. There are 7 early enzymatic HPV genes, and 2 late structural HPV genes.

Based on the discussion presented above, antisense/ribozyme therapeutics directed towards the HPV 6/11 groups may be constructed which target the viral E2 gene. It seems possible that the E2 gene target may be precarious with regard to the HPV 16/18 group, by a mechanism of driving integration of the virus through inhibition of E2 protein expression. Thus, it seems that the E6/E7 genes in HPV types 16/18 should be targeted directly.

Described below is the construction of antisense and ribozyme therapeutics into Sindbis virus vectors (described in Example 2) specific for HPV type 16 E6 and E7 RNA. Insertion of the HPV antisense and ribozyme moieties is between the Cla I and Xba I sites of the Sindbis vector.

#### A. CONSTRUCTION OF AN HPV 16 E6/E7 ANTISENSE THERAPEUTIC

The HPV 16 viral genomic clone, pHPV-16 (ATCC number 45113) is used as a template in a PCR reaction for the amplification of specific sequences from the viral E6/E7 genes. The HPV 16 antisense moiety is first inserted into the plasmid vector pKSII<sup>+</sup>; removal of the antisense therapeutic from the plasmid vector and insertion into the various Sindbis vector backbones is accomplished via the unique antisense moiety terminal Cla I and Xba I restriction endonuclease sites. Amplification of a portion of the HPV 16 E6/E7 genes is accomplished with the primer pair shown below:

Forward primer (buffer sequence/Xba I site/HPV 16 nucleotides 201-222):

---

YAYAYCYAGAGCAAGCAACAGTTACTGCGACG (SEQ. ID NO. 110)

---

Reverse primer (buffer sequence/Cla I site/HPV 16 nucleotides 759-738):

---

TATATATCGATCCGAAGCGTAGAGTCACACTTG (SEQ. ID NO. 111)

---

In addition to the HPV 16 E6/E7 complementary sequences, both primers contain a five nucleotide 'buffer sequences' at their 5' ends for efficient enzyme digestion of the PCR amplicon products. Generation of the HPV 16 amplicon with the primers shown above is accomplished with the PCR protocol described in Example 4. It has been shown previously that the E6/E7 mRNA in infected cervical epithelia is present in three forms, unspliced and two spliced alternatives (E6\* and E6\*\*), one in which nucleotides 226-525 of E6 are not present in the mature message (Smotkin et al., *J. Virol* 63:1441-1447, 1989). The region of complementary between the antisense moiety described here and the HPV 16 genome is viral nucleotides 201-759. Thus the antisense moiety will be able to bind to and inhibit the

## 153

translation of the E6/E7 unspliced message and the spliced E6\* and E6\*\* spliced messages.

The HPV 16 E6/E7 580 bp amplicon product is first purified with GENECLAN™, digested with the restriction enzymes Cla I and Xba I, and electrophoresed on a 1% agarose/TBE gel. The 568 bp band is then excised from the gel, the DNA purified with GENECLAN™ and ligated into the pKSII\* plasmid prepared by digestion with Cla I and Xba I, treatment with CIAP, and treatment with GENECLAN™. This plasmid is known as pKSaHPV16E6/E7.

#### B. CONSTRUCTION OF HPV 16 E6/E7 HAIRPIN RIBOZYME THERAPEUTICS

In order to efficiently inhibit the expression of HPV 16 E6 and E7 proteins, a hairpin ribozyme (HRBZ) with target specificities to E6 mRNA is constructed. The HPV 16 ribozyme moiety is first inserted into the plasmid vector pKSII\*; removal of the ribozyme therapeutic from the plasmid vector and insertion into the various Sindbis vector backbones is accomplished via the unique ribozyme moiety terminal Cla I and Xba I restriction endonuclease sites.

The HRBZ is homologous to the HPV 16 E6 RNA (nts 414-431) shown below:

---

TTAACTGTCAAAAGCCAC (SEQ. ID NO. 112)

---

The HRBZ is designed to cleave after the T residue in the TCTC hairpin ribozyme loop 5 substrate motif, shown underlined above. Following cleavage, the HRBZ is recycled and able to hybridize to, and cleave, another unspliced E6/E7 mRNA or the E6\* spliced mRNA molecule.

Double-stranded HRBZ as defined previously (Hampel et al., *Nucleic Acids Research* 18:299-304, 1990), containing a 4 base 'tetraloop' 3 and an extended helix 4, with specificity for the HPV 16 E6 RNA shown above, is chemically synthesized and includes both the 5' and 3' ends, respectively, Cla I and Xba I sites. The sequence of the chemically synthesized HPV 16 E6 HRBZ strands are shown below:

HPV 16 E6 HRBZ, sense strand (5'→3'):

---

5'-CGATGTGGCTTTTAGATGTTAAACAGAGAAACACACGGACTTCGGTC  
CGTGGTATATTAGCTGGTAT-3'  
(SEQ. ID NO. 113)

---

HPV 16 E6 HBRZ, antisense strand (5'→3'):

---

5'-CTAGATACCAGCTAATATACCACGGACCGAAGTCCGTGTTTCTCTGG  
TTTAACATCTAAAAGCCACAT-3' (SEQ. ID NO. 114)

---

In order to form the double-stranded HPV 16 E6 specific HRBZ with Cla I and Xba I cohesive ends, equal amounts of the oligonucleotides are mixed together in 10 mM Mg<sup>2+</sup>, heated at 95° C. for 5 minutes, then cooled slowly to room temperature to allow the strands to anneal.

The double-stranded HPV 16 E6 HRBZ with Cla I and Xba I cohesive ends is first ligated into the pKSII\* plasmid vector, prepared by digestion with Cla I and Xba I, treatment with CIAP, and treatment with GENECLAN™. This plasmid is known as pKSHPV16E6HRBZ.

## 154

The HPV 16 antisense and hairpin ribozyme moieties are liberated from their plasmid vectors, pKSaHPV16E6/E7 and pKSHPV16E6HRBZ, respectively, by digestion with Cla I and Xba I, purification by agarose electrophoresis and GENECLAN™, and insertion into the desired vector backbone, prepared by digestion with Cla I and Xba I, and treatment with CIAP. Several possible Sindbis vectors some of which are shown below, and whose detailed construction is described in Example 2, are suitable for the insertion of the HPV 16 antisense and ribozyme therapeutic moieties:

Vector	Functional Junction Region (+/-)
pKSSINBV	+
pKSSINBVdUR	-
pKSSINdURsjrc	+
pKSSINdURsjrPC	+
pKSSINdURsjrNP(7582-7601)	+
pKSSINdURsxjrc	+

Since the antisense and ribozyme therapeutic operate at the level of RNA, it is not necessary that the vectors containing these moieties contain a functional junction region. That is, translation of the region corresponding to the Sindbis structural proteins occurs only from subgenomic RNA. However, because translation of the antisense and hairpin ribozyme therapeutic is not an issue, these moieties will exert their affect from the level of positive stranded Sindbis genomic vector RNA.

On the other hand, it may be desired to administer repeated doses to an individual; thus the antisense and hairpin palliative would be inserted downstream of the adenovirus E3 or human cytomegalovirus H301 genes, which down-regulate the expression of MHC class I molecules in infected cells. Insertion of the antisense and hairpin palliatives is accomplished in the vectors from Examples 3 and 4 shown below, between the Cla I and Xba I sites:

Vector	Functional Junction Region (+/-)
pKSSINdURsjrcAdE3	+
pKSSINdURsjrcH301	+

Subgenomic mRNA is synthesized in these vectors, which serves as a translational template for the Ad E3 and CMV H301 genes. Thus, in these constructions, functional HPV 16 antisense and hairpin ribozyme palliatives will be present

on the levels of both subgenomic and positive stranded genomic Sindbis vector RNA.

Further, the HPV 16 antisense and hairpin ribozyme palliatives can be inserted downstream of a heterologous gene inserted into the described Sindbis vectors. For example, one could insert the HPV 16 antisense and hairpin ribozyme palliatives downstream of a heterologous gene coding for an immunogenic epitope of HPV 16 from, for example, the E6/E7 or L1 proteins. In these vectors, it would not be desired to include the immunoregulatory Ad E3 or CMV H301 genes.

Expression of the E6/E7 genes during infection with both the high- and low-risk HPV groups is required for proliferation of the cervical epithelium. The HPV E7 protein from all HPV types tested forms a complex with the retinoblastoma protein, and the E6 protein from HPV types 16 and 18 associates with and degrades the cellular p53 protein. The p53 and retinoblastoma cellular gene products are involved in the growth control of the cell, and altering the expression or function of these proteins can release the growth control in affected cells. Thus, an antisense or ribozyme therapeutic agent to both HPV groups should either directly or ultimately diminish the expression of one or both of these genes. Expression of the E6/E7 genes is trans-activated by the viral E2 protein. However, by utilizing an alternative splicing strategy, the E2 protein can also act as a trans-repressor. Integration of the oncogenic HPV types occurs in the viral E2 region and abrogates the expression of the E2 protein. Integration by the oncogenic HPV types appears to be a pivotal event in the frank induction and/or maintenance of cervical carcinoma. This event results in the constitutive expression of the E6/E7 genes. In the integrated state, expression of the E6/E7 genes is trans-activated by factors present in infected keratinocytes. The inactivation of the viral E2 control mechanism in response to the cellular keratinocyte factor activation of E6/E7 expression might be a critical event in viral integration.

#### Example 19

#### INHIBITION OF HUMAN INTERFERON A EXPRESSION IN INFECTED CELLS BY SEQUENCE-SPECIFIC RIBOZYME MOLECULES EXPRESSED FROM SINDBIS VIRUS VECTORS

Interferons (IFNs) comprise a family of small proteins which effect a wide range of biological activities in the mammalian cell, including the expression of MHC antigens, the expression of several genes which modulate cell growth control, and the resistance to viral infections (Pestka et al., *Ann. Rev. Biochem.* 56:727-777, 1987). Of the three classes of IFNs,  $\alpha$ ,  $\beta$ , and  $\gamma$ -IFN,  $\alpha$ -IFN, or leukocyte interferon, has a key role in limiting viral replication in the infected cell.

The antiviral effects of IFN- $\alpha$  are associated with the induction of two cellular enzymes which inhibit the viral lifecycle in the infected cell. One enzyme is a double-stranded RNA dependent 68-kDa protein kinase that catalyzes the phosphorylation of the  $\alpha$  subunit of the protein synthesis initiation factor eIF-2. The second enzyme induced by IFN- is 2',5'-oligoadenylate synthetase (2',5'-OAS), which in the presence of double-stranded RNA

activates the latent endonuclease, RNase L, which is responsible for degradation of viral and cellular RNAs (Johnston and Torrence, *Interferons* 3:189-298, Friedman (ed.), Elsevier Science Publishers, B.V., Amsterdam, 1984).

Because their replication strategy includes a double-stranded RNA intermediate, the RNA viruses in particular are strong inducers of interferon. With regard to Sindbis virus, double-stranded RNA molecules are present during the replication of both positive- and negative-stranded genome length molecules, and during the transcription of subgenomic mRNA. It has been demonstrated that infection of cells with Sindbis virus results in the induction of interferon (Saito, *J. Interferon Res.* 9:23-24, 1989).

In applications where extended expression of the therapeutic palliative is desired, expression of IFN in the infected cell is inhibited by inclusion of a hairpin ribozyme with specificity for IFN- $\alpha$  mRNA in the Sindbis vector. Inhibition of IFN- expression thus mitigates induction of the cascade of cellular proteins, including the eIF-2 protein kinase and 2',5'-OAS, which inhibit the extent to which virus can replicate in the infected cell. Prolonged expression of the therapeutic palliative without induction of an immune response targeted towards the vector infected cell is desired in all applications other than antigen presentation and includes, for example, systemic protein production, antisense and ribozyme, and accessory molecules.

#### A. CONSTRUCTION OF A HAIRPIN RIBOZYME WITH TARGETED SPECIFICITY FOR INTERFERON A mRNA

In order to efficiently inhibit the expression of interferon  $\alpha$  protein in cells infected with Sindbis vectors, a hairpin ribozyme (HRBZ) with target specificity for interferon  $\alpha$  mRNA is constructed. The IFN- $\alpha$  ribozyme moiety is first inserted into the plasmid vector pKSII<sup>+</sup> (Stratagene, La Jolla, Calif.); removal of the ribozyme therapeutic from the plasmid vector and insertion into the various Sindbis vector backbones is accomplished via the unique ribozyme moiety terminal Cla I and Xba I restriction endonuclease sites.

The HRBZ is homologous to nucleotides 1026-1041 of the human interferon alpha gene IFN-alpha 4b shown below, and to all IFN- $\alpha$  genes sequenced, including 5, 6, 7, 8, and 14, but not gene 16 (Henco et al., *J. Mol. Biol.* 185:227-260, 1985):

---

5'-TCT CTG TCC TCC ATG A  
(SEQ. ID NO. 120)

---

The HRBZ is designed to cleave after the T residue in the TGTC hairpin ribozyme loop 5 substrate motif, shown underlined above. Following cleavage, the HRBZ is recycled and able to hybridize to, and cleave, another IFN- $\alpha$  mRNA molecule.

Double-stranded HRBZ as defined previously (Hampel et al., *Nucleic Acids Research* 18:299-304, 1990), containing a 4 base tetraloop 3 and an extended helix 4, with specificity for the IFN- $\alpha$  mRNA shown above, is chemically synthesized and includes at the 5' and 3' ends, respectively, Cla I and Xba I sites. The sequence of the chemically synthesized IFN- $\alpha$  HRBZ strands are shown below:

IFN- $\alpha$  HRBZ, sense strand (5' to 3'):



TCG AGT CAT GGA GAG AGG AGA ACC AGA GAA ACA CAC GGA  
CTT CGG TCC GTG GTA TAT TAC CTG GAT  
(SEQ. ID NO. 121)

IFN- $\alpha$  HBRZ, antisense strand (5' to 3'):

CGA TCG AGG TAA TAT ACC ACG GAC CGA AGT CCG TGT GTT T  
CTCTG GTT C TC CTC TCT CCA TGA C  
(SEQ. ID NO. 122)

In order to form the double-stranded IFN- $\alpha$  specific HBRZ with Cla I and Xba I cohesive ends, equal amounts of the oligonucleotides are mixed together in 10 mM Mg2+, heated at 95° C. for 5 minutes, then cooled slowly to room temperature to allow the strands to anneal.

The double-stranded IFN- $\alpha$  HBRZ with Cla I and Xba I cohesive ends is first ligated into the pKSI<sup>+</sup> plasmid vector, prepared by digestion with Cla I and Xba I, treatment with CIAP, and treatment with GENECLAN™. This plasmid is known as pKSI<sup>+</sup>NaHRBZ.

The IFN- $\alpha$  hairpin ribozyme moiety is liberated from the pKSI<sup>+</sup>NaHRBZ plasmid by digestion with Cla I and Xba I, purification by 2% Nu-Sieve/1% agarose electrophoresis and GENECLAN™, and insertion into the desired vector backbone, prepared by digestion with Cla I and Xba I, and treatment with CIAP. Several possible Sindbis vectors some of which are shown below, and whose detailed construction is described in Examples 2, 3, and 4 are suitable for the insertion of the IFN- $\alpha$  hairpin ribozyme moiety:

Vector	Functional Junction Region (+/-)
pKSSINBV	+
pKSSINBVdUR	-
pKSSINdURsjrc	+
pKSSINdURsjrcPC	+
pKSSINdURsjrcNP(7582-7601)	+
pKSSINdURsjrcE3	+
pKSSINdURsjrcAdE3	+
pKSSINdURsjrcH301	+

Since the ribozyme activity operates at the level of RNA, it is not necessary that this region is expressed as a portion subgenomic mRNA. However, when placed downstream of a functional junction region, the level of ribozyme synthesized is much greater and perhaps more effective in cleaving the IFN- $\alpha$  RNA target.

Further, in some applications, for example systemic expression of protein, multiple dose administration to an individual is required. In these applications, prolonged expression of the therapeutic palliative without induction of an immune response targeted towards the vector infected cell is desired. In this configuration, the IFN- $\alpha$ HRBZ moiety could be inserted upstream of the adenovirus E3 or human cytomegalovirus H301 genes, which down-regulate the expression of MHC class I molecules in infected cells. Following the gene which modulates MHC class I expression is, consecutively, an IRES element selected from among the group described in Example 5, and the therapeutic palliative. Ordered insertion of the hairpin ribozyme, Ad E3 or CMV H301, IRES, and heterologous gene of interest components along the multiple cloning sequence located in the vector between the vector junction region and 3' end is

accomplished by modification with the appropriate restric-

tion enzyme recognition sites of the component 5' and 3' ends. In these constructions, functional INF- $\alpha$  hairpin ribozyme palliatives will be present at the level of both subgenomic and positive stranded genomic Sindbis vector RNA.

#### Example 20

### EX VIVO AND IN VIVO TREATMENT OF HUMAN CANCERS BY ADMINISTRATION OF RECOMBINANT ALPHAVIRUS VECTOR PARTICLES OR ALPHAVIRUS PLASMID DNA VECTORS WHICH EXPRESS CYTOKINES, CYTOKINE RECEPTORS, OR DRUG POTENTIATORS

#### A. VECTOR CONSTRUCTIONS

##### 1. GAMMA INTERFERON

Murine gamma interferon is subcloned from the retroviral vector plasmid pMu- $\gamma$ IFN (Howard et al., *Ann. N.Y. Acad. Sci.* 716:167-187, 1994) by digesting with Cla I and making the termini blunt by Klenow enzyme and dNTPs. After heat inactivation of the Klenow enzyme, the vector is digested with Xho I. The resulting 800 bp fragment containing the coding sequences of gamma interferon is isolated from a 1% agarose gel. pKSSINBV (Example 3) is digested with Xho I and Stu I, and the vector is purified by GENECLAN™ and ligated with the gamma interferon insert. The resulting vector construction is known as pKSSIN $\gamma$ Mu. The human gamma interferon gene (Howard et al., *supra*) is similarly inserted into pKSSINBV using the same strategy. The resulting vector construct is known as pKSSIN $\gamma$ Hu. The interferon expressing Sindbis vectors are then packaged into vector particles. This is accomplished by introducing RNA from these vectors into a packaging cell line as described in Example 7.

The mouse and human interferon genes are also cloned into pVGELVISSINBV-linker (see Example 3). Briefly, pVGELVISSINBV-linker is first digested with Asc I and the termini made blunt by the addition of Klenow enzyme and dNTPs. The Klenow is heat inactivated and the vector is subsequently digested with Xho I. This vector is purified by GENECLAN™ and ligated to the gamma interferon inserts prepared as described above. The resulting vectors are described pVGELVIS- $\gamma$ Mu and pVGELVIS- $\gamma$ Hu, respectively.

##### 2. INTERLEUKIN-2

The human IL-2 gene is cloned by PCR amplification into the KT-3 retroviral backbone (Howard et al., *Ann. N.Y. Acad. Sci.* 716:167-187, 1994). The source for the IL-2 gene is a pBR322 based plasmid which contains the IL-2 cDNA (ATCC #61391). The cDNA is PCR amplified using a standard three-temperature protocol as described in Example 3. The 5' primer is the sense sequence of the hIL-2

gene complementary to the 5' coding region beginning at the ATG start codon. Additionally, a Xho I site is built into the 5' end of the primer sequence.

5'hIL-2 (SEQ. ID NO. 123)

5'-GCCTCGAGACAATGTACAGGATGCAACTCCTGTCT

The 3' primer is an antisense sequence of the hIL-2 gene complementary to the 3' coding region ending at the TAA stop codon. Additionally, a Cla I site is built into the 5' end of the primer sequence.

3'hIL-2(SEQ. ID NO. 124)

5'-GAATCGATTATCAAGTCAGTGTGGAGATGATGCT

The PCR amplicon is purified in a 1% agarose gel. To place the IL-2 gene in the KT-3 retroviral backbone, pMu-IFN is digested with Xho I and Cla I to remove the interferon gene. After treatment with phosphatase, the vector is purified in a 1% agarose gel. The vector and IL-2 insert are ligated and transformed using standard procedures, and recombinant clones are screened by restriction enzyme analysis. The resulting vector is designated pKThIL-2.

Human IL-2 is subcloned from the retroviral vector pKThIL2, into the pKSSINBV vector, using the same strategy employed for murine gamma interferon. The resulting vector construction is known as pKSSIN-huIL-2. The human IL-2 gene is also cloned into pVGELVISSINBV-linker as described above for the gamma interferon genes. The resulting construct is designated pVGELVIS-IL-2.

### 3. HSV-TK

The coding region and transcriptional termination signals of HSV-1 thymidine kinase gene (HSV-TK) are isolated as a 1.8 kb BglII/Pvu II fragment from plasmid 322TK (McKnight et. al., *Nuc. Acids Res.* 8:5949, 1980) cloned into pBR 322 (ATCC No. 31344). The ends are made blunt by the addition of Klenow enzyme and dNTPs. The 1.8 kb fragment is isolated on a 1% agarose gel and ligated to pKSSINBV which had been previously digested with Stu I, phosphatased and gel purified. This construct is known as pKSSINBV-TK. For use in physical gene transfer experiments, the TK gene is similarly cloned into pVGELVIS-SINBV-linker. The vector is prepared by digestion with Pml I, phosphatase treatment and isolated on a 1% agarose gel. This vector construct is known as pVGELVISBV-TK.

### B. ADMINISTRATION

Any of the above-described vector constructs may be utilized along with packaging cell lines described in Example 7, in order to produce recombinant alphavirus particles suitable for administration to humans or animals (either directly or indirectly), or for infecting target cells. Such vector constructs may also introduced directly into target cells as a "naked" DNA molecule, as a DNA complex with various liposome formulations, or as a DNA ligand complex including the alphavirus DNA vector molecule (e.g., along with a polycation compound such as polylysine, a receptor specific ligand, or a psoralen inactivated virus such as Sendai or Adenovirus).

This aspect of the invention relates to pharmaceutical compositions comprising alphavirus vector constructs, recombinant alphavirus particles, or eukaryotic layered vector initiation systems described above (individually and/or collectively referred to herein sometimes as "gene delivery vehicles"), in combination with a pharmaceutically acceptable carrier or diluent. Such gene delivery vehicles can be

formulated in crude or, preferably, purified form. Pharmaceutical compositions comprising the gene delivery vehicles may be prepared either as a liquid solution or as a solid form (e.g., lyophilized) which is resuspended in a solution prior to administration. In addition, the composition may be prepared with suitable carriers or diluents for topical administration, injection, or nasal, oral, vaginal, sub-lingual, inhalant, intraocular, enteric, or rectal administration.

Pharmaceutically acceptable carriers or diluents are non-toxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic saline solutions, preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin (HSA).

Gene delivery vehicles according to the invention can be stored in liquid, or preferably, lyophilized form. Factors influencing stability include the formulation (liquid, freeze dried, constituents thereof, etc.) and storage conditions, including temperature, storage container, exposure to light, etc. Alternatively, pharmaceutical compositions according to the invention can be stored as liquids at low temperatures. In a preferred embodiment, the gene delivery vehicles of the invention are formulated to preserve infectivity in a lyophilized form at elevated temperatures, and for this form to be suitable for injection into patients following reconstitution.

In another aspect of the present invention, methods are provided for preventing or treating various diseases and genetic disorders. Such methods comprise administering a gene delivery vehicle as described above, such that a therapeutically efficacious amount of the desired, or "selected," gene product is produced. As used herein, a "therapeutically effective amount" is an amount that is of clinical relevance, i.e., protective immunity is achieved, tumor progression is retarded, etc. A "therapeutically effective amount" of a gene delivery vehicle according to the invention refers to the amount that must be administered to produce a therapeutically effective amount of the desired gene product in a particular patient or application. For instance, in a patient suffering from hemophilia A, a therapeutically effective amount of a gene delivery vehicle is an amount that elicits production of sufficient factor VIII (the desired gene product expressed from the selected heterologous nucleotide sequence) to produce therapeutically beneficial clotting and will thus generally be determined by each patient's attending physician, although serum levels of about 0.2 ng/mL (about 0.1% of "normal" levels) or more will be therapeutically beneficial. Typical dosages will range from about  $10^5$  to  $10^{12}$  gene delivery vehicles.

In some cases, gene delivery vehicles according to the invention will be administered as an adjunct to other therapy, such as hormonal, radiation, and/or chemotherapeutic treatment.

In various embodiments of the invention, gene delivery vehicles may be administered by various routes in vivo, or ex vivo, as described in greater detail below. Alternatively, the gene delivery vehicles of the present invention may also be administered to a patient by a variety of other methods. Representative examples include transfection by various physical methods, such as lipofection (Felgner, et al., *Proc. Natl. Acad. Sci. USA*, 84:7413, 1989), direct DNA injection (Acsadi, et al., *Nature*, 352:815, 1991; microprojectile bombardment (Williams, et al., *Proc. Nat'l. Acad. Sci. USA*, 88:2726, 1991); liposomes of several types (see e.g., Wang, et al., *Proc. Nat'l. Acad. Sci. USA*, 84:7851, 1987); CaPO<sub>4</sub>

(Dubensky, et al., *Proc. Nat'l. Acad. Sci. USA*, 81:7529, 1984); DNA ligand (Wu, et al., *J. Biol. Chem.*, 264:16985, 1989); or administration of nucleic acids alone (WO 90/11092). Other possible methods of administration can include injection of producer cell lines into the blood or, alternatively, into one or more particular tissues, grafting tissue comprising cells treated with gene delivery vehicles according to the invention, etc.

When pharmaceutical compositions according to the invention are administered in vivo, i.e., to the cells of patient without prior removal of the cells from the patient, administration can be by one or more routes. In this context, "administration" is equivalent to "delivery." Typical routes of administration include traditional parenteral routes, such as intramuscular (i.m.), subcutaneous (sub-q), intravenous (i.v.), and interperitoneal (i.p.) injection. Other suitable routes include nasal, pulmonary, and even direct administration into a particular tissue, such as the liver, bone marrow, etc. In addition, other routes may be employed, as described below.

Transdermal or topical application of a pharmaceutical composition comprising a gene delivery vehicle according to the invention may be used as an alternate route of administration because the skin is the most expansive and readily accessible organ of the human body. Transdermal delivery systems (TDS) are capable of delivering a gene delivery vehicle through intact skin so that it reaches the systemic circulation in sufficient quantity to be therapeutically effective. TDS provide a variety of advantages, including elimination of gastrointestinal absorption problems and hepatic first pass effect, reduction of dosage and dose intervals, and improved patient compliance. The major components of TDS are a controlled release device composed of polymers, a gene delivery vehicle according to the invention, excipients, and enhancers, and a fastening system to fix the device to the skin. A number of polymers have been described and include, but are not limited to, gelatin, gum arabic, paraffin waxes, and cellulose acetate phthalate (Sogibayasi, et al., *J. Controlled Release*, 29:177, 1994). These polymers can be dermatologically formulated into aqueous, powder, or oil phases. Various combinations can produce lotions, pastes, ointments, creams, and gels, alone or together with the aid of emulsifiers.

Additionally, iontophoresis may be used to cause increased penetration of ionized substances into or through the skin by the application of an electrical field. This method has the advantage of being able to deliver the drug in a pulsatile manner (Singh, et al., *Dermatology*, 187:235, 1993).

Topical administration may also be accomplished by encapsulating gene delivery vehicles according to the invention in liposomes. Hyaluronic acid has been used as a bioadhesive ligand for the formation of liposomes to enhance adherence and retention to the extracellular matrix in cases of burns and wound healing (Yerushalmi, et al., *Arch. Biochem. and Biophys.*, 313:267, 1994). As those in the art will appreciate, methods of liposome preparation can be tailored to control size and morphology. Liposomes can also be made to include one or more targeting elements to target a specific cell type.

Ocular administration is an alternate route to achieve delivery of compositions described herein. Systemic absorption occurs through contact with the conjunctival and nasal mucosae, the latter occurring as the result of drainage through the nasolacrimal duct. Formulations such as those described above which further comprise inert ingredients such as buffers, chelating agents, antioxidants, and preservatives can be incorporated into ophthalmic dosage forms

intended for multiple dose use. Formulations also may consist of aqueous suspensions, ointments, gels, inserts, bioadhesives, microparticles, and nanoparticles.

The nasal cavity also offers an alternative route of administration for compositions comprising a gene delivery vehicle as described herein. For instance, the human nasal cavities have a total surface area of approximately 150 cm<sup>2</sup> and are covered by a highly vascular mucosal layer. A respiratory epithelium, comprised of columnar cells, goblet cells, and ciliary cuboidal cells, lines most of the nasal cavity (Chien, et al., *Crit. Rev. in Therap. Drug Car. Sys.*, 4:67, 1987). The subepithelium contains a dense vascular network and the venous blood from the nose passes directly into the systemic circulation, avoiding first-pass metabolism in the liver. Thus, delivery to the upper region of the nasal cavity may result in slower clearance and increased bioavailability of gene delivery vehicles. The absence of cilia in this area is an important factor in the increased effectiveness of nasal sprays as compared to drops. The addition of viscosity-building agents, such as methylcellulose, etc. can change the pattern of deposition and clearance of intranasal applications. Additionally, bioadhesives can be used as a means to prolong residence time in the nasal cavity. Various formulations comprising sprays, drops, and powders, with or without the addition of absorptive enhancers, have been described (see Wearley, L, supra).

Oral administration includes sublingual, buccal, and gastrointestinal delivery. Sublingual and buccal (cheek) delivery allow for rapid systemic absorption of gene delivery vehicles and avoid hepatic first-pass metabolism and degradation in the stomach and intestines. Unidirectional buccal delivery devices can be designed for oral mucosal absorption only. Additionally, these devices can prevent diffusion-limiting mucus buildup to allow for enhanced absorption. Delivery through the gastrointestinal tract allows for precise targeting for drug release. Depending on the formulation, gene delivery vehicles can be specifically delivered to areas in the stomach, duodenum, jejunum, ileum, cecum, colon, or rectum. Oral formulations include tablets, capsules, aqueous suspensions, and gels. These may contain bioadhesive polymers, hydrodynamically balanced systems, gastroinflatable delivery devices, intragastric retention shapes, enteric coatings, excipients, or intestinal absorption promoters (Ritschel, W. A., *Meth. Exp. Clin. Pharmacol.*, 13:313, 1991).

The human rectum has a surface area of between 200 to 400 cm<sup>2</sup> and is abundant in blood and lymphatic vessels. This offers an alternative route for administering compositions according to the invention. Depending on the actual site of administration, it may be possible to bypass first-pass metabolism by the liver. Targeting of the systemic circulation can be achieved by delivering the vehicle to an area behind the internal rectal sphincter which allows absorption directly into the inferior vena cava, thereby bypassing the portal circulation and avoiding metabolism in the liver. The liver can be targeted by delivering the vehicle to the region of the ampulla recti, which allows absorption into the portal system (Ritschel, supra).

Alternatively, pulmonary administration can be accomplished through aerosolization. As the lungs are highly vascularized, this type of administration allows systemic delivery. The three systems commonly used for aerosol production are: the nebulizer, the pressurized metered dose inhaler, and the dry powder inhaler, all of which are known in the art. Aerosol therapy is very common in obstructive bronchial diseases but can be used as well as for the treatment of systemic diseases. The surface area of the adult

human lung is approximately 75 m<sup>2</sup> and requires only one puff of an aerosol to cover this entire area within seconds. Absorption occurs quickly because the walls of the alveoli in the deep lung are extremely thin. Absorption and clearance depends on a number of factors, including particle size and solubility (Wearley, L, supra). Particles are preferably smaller than 5 µm in diameter.

The vaginal mucosa consists of stratified squamous epithelium. Gene delivery vehicles can be administered through the vaginal orifice onto the mucosa. Formulations include ointments, creams, and suppositories. Additional information regarding these and other routes of administration may be found in U.S. Ser. No. 08/366,788.

As an alternative to in vivo administration of the gene delivery vehicles of the invention, ex vivo administration can be employed. Ex vivo treatment envisions withdrawal or removal of a population of cells from a patient. Exemplary cell populations include bone marrow cells, liver cells, and blood cells from the umbilical cord of a newborn. Such cells may be processed to purify desired cells for transduction prior to such procedures, for instance to obtain subsets of such cell populations, e.g., CD34<sup>+</sup> bone marrow progenitor cells. Preferred methods of purification include various cell sorting techniques, such as antibody panning, FACS, and affinity chromatography using a matrix coupled to antibodies specifically reactive to the desired cell type(s). Isolated cells are then transduced, after which they may be immediately re-introduced to the patient from which they were withdrawn. Alternatively, the cells may be expanded in culture by various techniques known to those skilled in the art prior to re-introduction.

In another embodiment of the invention, gene delivery vehicles of the invention are administered to patients in conjunction with another therapeutic compound. As those in the art will appreciate, such compounds may include, but are not limited to, other gene delivery vehicles designed to deliver one or more other therapeutic genes to the patient, as is described in U.S. Ser. No. 08/368,210, U.S. Ser. No. 08/368,210.

In accordance with the non-parenteral administration the present invention, the gene delivery vehicles, particularly those comprised of unencapsidated nucleic acid, may be complexed with a polycationic molecule to provide polycation-assisted non-parenteral administration. Such a method of gene delivery facilitates delivery of a gene via mediation by a physical particle comprised of multiple components that augment the efficiency and specificity of the gene transfer. In particular, polycationic molecules, such as polylysine and histone, have been shown to neutralize the negative charges on a nucleic acid molecule and to condense the molecule into a compact form. This form of molecule is transferred with high efficiency in cells, apparently through the endocytic pathway. The uptake in expression of the nucleic acid molecule in the host cell results after a series of steps, as follows: (1) attachment to cell surface; (2) cell entry via endocytosis or other mechanisms; (3) cytoplasmic compartment entry following endosome release; (4) nuclear transport; and (5) expression of the nucleic acid molecule carried by the gene delivery vehicle. In a further preferred embodiment, multi-layer technologies are applied to the polycation-nucleic acid molecule complex to facilitate completion of one or more of these steps. For example, a ligand such as asialoglycoprotein, transferrin, and immunoglobulin may be added to the complex to facilitate binding of the cell complex to the cell surface, an endosomal disruption component (e.g., a viral protein, a fusogenic peptide such as the n-terminus of the influenza virus hema-

glutinin or an inactivated virus) is added to facilitate the release of DNA from the endosome, or a nuclear protein (or a peptide containing a nuclear localization signal) is added to facilitate the transport of the DNA into the nucleus. In a further preferred embodiment, the composition comprising the complex includes inactivated adenovirus particles (Curiel, D. T., et al., *PNAS* 88: 8850-8854, 1991; Cristiano, R. J., *PNAS* 90: 2122-2126 1993; Cotten, M., et al., *PNAS* 89: 6094-6098 1992; Lozier, J. N., et al., *Human Gene Therapy* 5: 313-322, 1994; Curiel, D. T., et al., *Human Gene Therapy* 3: 147-154, 1992; Plank, C. et al., *Bioconjugate Chem.* 3: 533-539, 1992; Wagner, E. et al., *PNAS* 88: 4255-4259, 1991). The assorted components comprising the multi-layer complex may be varied as desired, so that the specificity of the complex for a given tissue, or the gene expressed from the gene delivery vehicle, may be varied to better suit a particular disease or condition.

As noted above, various methods may be utilized to administer gene delivery vehicles of the present invention, including nucleic acids which encode the immunogenic portion(s) discussed above, to warm-blooded animals such as humans, directly. Suitable methods include, for example, various physical methods such as direct DNA injection (Acscadi et al., *Nature* 352:815-818, 1991), and microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991).

Within an in vivo context, the gene delivery vehicle can be injected into the interstitial space of tissues including muscle, brain, liver, skin, spleen or blood (see, WO 90/11092). Administration may also be accomplished by intravenous injection or direct catheter infusion into the cavities of the body (see, WO 93/00051), discussed in more detail below.

It is generally preferred that administration of the gene delivery vehicles at multiple sites be via at least two injections. In this regard, suitable modes of administration include intramuscular, intradermal and subcutaneous injections, with at least one of the injections preferably being intramuscular. In particularly preferred embodiments, two or more of the injections are intramuscular. However, although administration via injections is preferred, it will be evident that the gene delivery vehicles may be administered through multiple topical or separate ocular administrations. Further, a number of additional routes are suitable for use within the present invention when combined with one or more of the routes briefly noted above, including intraperitoneal, intracranial, oral, rectal, nasal, vaginal and sublingual administration. Methods of formulating and administering the gene delivery vehicles at multiple sites through such routes would be evident to those skilled in the art and are described in U.S. Ser. No. 08/366,788 and U.S. Ser. No. 08/367,071 incorporated herein by reference in their entirety.

#### C. Liposome Formulation

Several methods may be used in the preparation of liposomes to incorporate gene delivery vehicles of the invention, particularly those that are DNA or RNA, see Gregoriadis et. al., (*Liposome Technology*, CFC Press, New York 1984), Ostro et. al., (*Liposomes*, Marek Dekker, 1987) and Lichtenberg et. al., (*Meth. Biochem. Anal.* 33:337, 1988). According to one embodiment of the invention, the gene delivery vehicles are complexed with cationic liposomes or lipid vesicles. Cationic liposome formulations may be prepared from a mixture of positively charged lipids, negatively charged lipids, neutral lipids and cholesterol or similar sterol. The positively charged lipids may be DMRIE (Felgner, et. al., *J. Biol. Chem.* 269:1, 1994), DOTMA,

DOTAP or analogs thereof or a combination of two or more of these lipids. DMRIE is described in U.S. Ser. No. 07/686,746 which is hereby incorporated reference. The neutral and negatively charged lipids can be any natural or synthetic phospholipid or mono-, di- or triglycerols. The natural phospholipids may be derived from animal and plant sources. For example, natural phospholipids such as phosphatidylcholine, phosphatidylethanolamine, sphingomylin, phosphatidylserine, or phosphatidylinositol may be utilized. Synthetic phospholipids may be selected from those having fatty acid groups such as dimyristoylphosphatidylcholine, distearoylphosphatidylcholine, dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, and the corresponding phosphatidylethanolamines and phosphatidylglycerols. The neutral lipids may be phosphatidylcholine, cardiolipin, phosphatidylethanolamine, mono-, di- or triacylglycerols, or analogs thereof such as dioleoylphosphatidylethanolamine (DOPE). The negatively charged lipids may be phosphatidylglycerol, phosphatidic acid or a similar phospholipid analog. Other additive known to those skilled in the art may also be used such as cholesterol, glycolipids, fatty acids, sphingolipids, prostaglandins, gangliosides, neobee, niomes, or any other natural or synthetic amphophiles.

Substitution of the cationic lipid component of liposomes may be used to alter the transfection efficiency of the

liposome. For example, 1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DMRIE) is used in conjunction with DOPE which provides increased transfection efficiency and does not aggregate at high concentrations as other formulations such as DC-cholesterol/DOPE. These characteristics allows for higher absolute concentrations of DNA and liposomes to be introduced into patients in vivo without increased levels of toxicity. A preferred molar ratio of DMRIE to DOPE of 9:1 to 1:9 with a particularly preferred molar ratio of 5:5 (see WO 94/29469 incorporated herein by reference)

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Additionally, the publications and other materials cited to illuminate the background of the invention, and in particular, to provide additional details concerning its practice as described in the detailed description and examples, are hereby incorporated by reference in their entirety.

A Sequence Listing has also been included herewith in accordance with the provisions of 37 C.F.R. § 1.821 et seq. To the extent any discrepancy exists between the Specification Figures and the Sequence Listing, the specification or Figures should be considered to be the primary document.

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#### SEQUENCE LISTING

##### ( 1 ) GENERAL INFORMATION:

( i i i ) NUMBER OF SEQUENCES: 128

##### ( 2 ) INFORMATION FOR SEQ ID NO:1:

###### ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 16656 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

###### ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATTGACGGCG TAGTACACAC TATTGAATCA AACAGCCGAC CAATCGCACT ACCATCACAA	60
TGGAGAAGCC AGTAGTAAAC GTAGACGTAG ACCCCCAGAG TCCGTTTGTC GTGCAACTGC	120
AAAAAAGCTT CCGCAATTT GAGGTAGTAG CACAGCAGGT CACTCCAAAT GACCATGCTA	180
ATGCCAGAGC ATTTTCGCAT CTGGCCAGTA AACTAATCGA GCTGGAGGTT CCTACCACAG	240
CGACGATCTT GGACATAGGC AGCGCACCGG CTCGTAGAAT GTTTTCCGAG CACCAGTATC	300
ATTGTGTCTG CCCCATGCGT AGTCCAGAAG ACCCGGACCG CATGATGAAA TATGCCAGTA	360
AACTGGCGGA AAAAGCGTGC AAGATTACAA ACAAGAACTT GCATGAGAAG ATTAAGGATC	420
TCCGGACCGT ACTTGATACG CCGGATGCTG AAACACCATC GCTCTGCTTT CACAACGATG	480
TTACCTGCAA CATGCGTGCC GAATATTCCG TCATGCAGGA CGTGATATATC AACGCTCCCG	540
GAACATCTA TCATCAGGCT ATGAAAGGCG TCGGGACCCT GTACTGGATT GGCTTCGACA	600
CCACCCAGTT CATGTTCTCG GCTATGGCAG GTTCGTACCC TCGGTACAAC ACCAACTGGG	660
CCGACGAGAA AGTCCTTGAA GCGCGTAACA TCGGACTTTG CAGCACAAAG CTGAGTGAAG	720
GTAGGACAGG AAAATTGTCG ATAATGAGGA AGAAGGAGTT GAAGCCCGGG TCGCGGGTTT	780
ATTTCTCCGT AGGATCGACA CTTTATCCAG AACACAGAGC CAGCTTGCAAG AGCTGGCATC	840

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CAGTAAAAGG	AGAACGGGTA	TCGTTCCCTG	TGTGCACGTA	CATCCCGGCC	ACCATATGCG	1080
ATCAGATGAC	TGGTCTAATG	GCCACGGATA	TATCACCTGA	CGATGCACAA	AAACTTCTGG	1140
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GCGTAAAAGT	CCCAGCCTCT	TTTAGCGCTT	TCCCCATGTC	GTCCGTATGG	ACGACCTCTT	1440
TGCCCATGTC	GCTGAGGCAG	AAATTGAAAC	TGGCATTGCA	ACCAAAGAAG	GAGGAAAAAC	1500
TGCTGCAGGT	CTCGGAGGAA	TTAGTCATGG	AGGCCAAGGC	TGCTTTTGAG	GATGCTCAGG	1560
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CGGTGCTCGA	CACGTCGAAA	GAGGAACAAC	TCAAACCTCAG	GTACCAGATG	ATGCCCACCG	5940
AAGCCAACAA	AAGTAGGTAC	CAGTCTCGTA	AAGTAGAAAA	TCAGAAAGCC	ATAACCACTG	6000
AGCGACTACT	GTCAGGACTA	CGACTGTATA	ACTCTGCCAC	AGATCAGCCA	GAATGCTATA	6060
AGATCACCTA	TCCGAAACCA	TTGTACTCCA	GTAGCGTACC	GGCGAACTAC	TCCGATCCAC	6120
AGTTTCGTGT	AGCTGTCTGT	AACAACATATC	TGCATGAGAA	CTATCCGACA	GTAGCATCTT	6180
ATCAGATTAC	TGACGAGTAC	GATGCTTACT	TGGATATGGT	AGACGGGACA	GTCGCCCTGCC	6240
TGGATACTGC	AACCTTCTGC	CCCGCTAAGC	TTAGAAGTTA	CCCGAAAAAA	CATGAGTATA	6300
GAGCCCCGAA	TATCCGCAGT	GCGGTTCCAT	CAGCGATGCA	GAACACGCTA	CAAAATGTGC	6360
TCATTGCCGC	AACTAAAAGA	AATTGCAACG	TCACGCAGAT	GCGTGAACTG	CCAACACTGG	6420
ACTCAGCGAC	ATTCAATGTC	GAATGCTTTC	GAAAATATGC	ATGTAATGAC	GAGTATTGGG	6480
AGGAGTTCGC	TCGGAAGCCA	ATTAGGATTA	CCACTGAGTT	TGTCACCGCA	TATGTAGCTA	6540
GA CTGAAAGG	CCCTAAGGCC	GCCACACTAT	TTGCAAAGAC	GTATAATTTG	GTCCCATTGC	6600
AAGAAGTGCC	TATGGATAGA	TTCGT CATGG	ACATGAAAAG	AGACGTGAAA	GTTACACCAG	6660
GCACGAAACA	CACAGAAGAA	AGACCGAAAAG	TACAAGTGAT	ACAAGCCGCA	GAACCCCTGG	6720
CGACTGCTTA	CTTATGCGGG	ATTCACCGGG	AATTAGTGCG	TAGGCTTACG	GCCGTCTTGC	6780
TTCCAAACAT	TCACACGCTT	TTTGACATGT	CGGCGGAGGA	TTTTGATGCA	ATCATAGCAG	6840
AACACTTCAA	GCAAGGCGAC	CCGGTACTGG	AGACGGATAT	CGCATCATTC	GACAAAAGCC	6900
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CACTACTCGA	CTTGATCGAG	TGCGCCTTTG	GAGAAATATC	ATCCACCCAT	CTACCTACGG	7020
G TACTCGTTT	TAAATTGCGG	GCGATGATGA	AATCCGGAAT	GTTCTCTACA	CTTTTTGTCA	7080
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GCCCTTTCCC	GGCCCCCACT	GCCATGTGGA	GGCCGCGGAG	AAGGAGGCAG	GCGGCCCCGA	7740
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CCGACAGATT	GTTTCGACGTC	AAGAACGAGG	ACGGAGATGT	CATCGGGCAC	GCACTGGCCA	8040



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GAGGAGACAG	CGGTGCTCCG	ATCATGGATA	ACTCCGGTCG	GGTTGTGCGG	ATAGTCCTCG	8340
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GCGAACCTTC	CAGAGCCCTC	GACATCCTTG	AAGAGAACGT	GAACCATGAG	GCCTACGATA	8580
CCCTGCTCAA	TGCCATATTG	CGGTGCGGAT	CGTCTGGCAG	AAGCAAAAGA	AGCGTCGTTG	8640
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GTACACTGGC	CCGCAAGATA	AAACCAAAAT	TCGTGGGACG	GGAAAAATAT	GATCTACCTC	9060
CCGTTACAGG	TAAAAAGAATT	CCTTGACACAG	TGTACGACCG	TCTGAAAACA	ACTGCAGGCT	9120
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GGAAAGTTTA	CGCAAAGCCG	CCATCTGGGA	AGAACATTAC	GTATGAGTGC	AAGTGCGGCG	9240
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AGTGCGTCGC	CTATAAGAGC	GACCAAAACGA	AGTGGGTCTT	CAACTCACCG	GACTTGATCA	9360
GACATGACGA	CCACACGGCC	CAAGGGAAAT	TGCATTTGCC	TTTCAAGTTG	ATCCCGGGTG	9420
CCTGCATGGT	CCCTGTTGCC	CACGCGCCGA	ATGTAATACA	TGGCTTTAAA	CACATCAGCC	9480
TCCAATTAGA	TACAGACCAC	TTGACATTGC	TCACCACCAG	GAGACTAGGG	GCAAACCCGG	9540
AACCAACCAC	TGAATGGATC	GTCGGAAAAG	CGGTCAGAAA	CTTCACCGTC	GACCGAGATG	9600
GCCTGGAATA	CATATGGGGA	AATCATGAGC	CAGTGAGGGT	CTATGCCCAA	GAGTCAGCAC	9660
CAGGAGACCC	TCACGGATGG	CCACACGAAA	TAGTACAGCA	TTACTACCAT	CGCCATCCTG	9720
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CAGTGTTATG	TGCCTGTAAA	GCGCGCCGTG	AGTGCCTGAC	GCCATACGCC	CTGGCCCCAA	9840
ACGCCGTAAT	CCCAACTTCG	CTGGCACTCT	TGTGCTGCGT	TAGGTCGGCC	AATGCTGAAA	9900
CGTTCACCGA	GACCATGAGT	TACTTGTGGT	CGAACAGTCA	GCCGTTCTTC	TGGGTCCAGT	9960
TGTGCATACC	TTTGGCCGCG	TTCATCGTTC	TAATGCCTCA	CTGCTCCTGC	TGCCTGCCTT	10020
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TCAATTTGGA	GATCACTGTC	ATGTCCTCGG	AGGTTTTGCC	TTCCACCAAC	CAAGAGTACA	10200
TTACCTGCAA	ATTCAACACT	GTGGTCCCTT	CCCCAAAAAT	CAAATGCTGC	GGCTCCTTGG	10260
AATGTCAGCC	GGCCGCTCAT	GCAGACTATA	CCTGCAAGGT	CTTCGGAGGG	GTCTACCCCT	10320
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ACGTGGAATT	GTCAGCAGAT	TGCGCGTCTG	ACCACGCGCA	GGCGATTAAG	GTGCACACTG	10440

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CAGCATCGTT	TACGCCATTG	GATCATAAGG	TCGTTATCCA	TCGCGGCCTG	GTGTACAAC	10620
ATGACTTCCC	GGAATATGGA	GCGATGAAAC	CAGGAGCGTT	CGGAGACATT	CAAGCTACCT	10680
CCTTGACTAG	CAAGGATCTC	ATCGCCAGCA	CAGACATTAG	GCTACTCAAG	CCTTCGCGCA	10740
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CGACCAGCAA	AACTCGATGT	ACTTCCGAGG	AACTGATGTG	CATAATGCAT	CAGGCTGGTA	11460
CATTAGATCC	CCGCTTACCG	CGGGCAATAT	AGCAACACTA	AAAACCTCGAT	GTACTTCCGA	11520
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CTAGCGGACG	CCAAAAACTC	AATGTATTTT	TGAGGAAGCG	TGGTGCATAA	TGCCACGCAG	11640
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AAAAAAAAAA	AAAAAAAAAA	AAAAATCTAG	AGGGCCCTAT	TCTATAGTGT	CACCTAAATG	11760
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CGGGCTTTCC	CCGTCAAGCT	CTAAATCGGG	GCATCCCTTT	AGGGTTCCGA	TTTAGTGCTT	12240
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GCTCCCCAGC	AGGCAGAAAG	ATGCAAAAGCA	TGCATCTCAA	TTAGTCAGCA	ACCATAGTCC	12660
CGCCCCCTAA	TCCGCCCATC	CCGCCCTTAA	CTCCGCCCAAG	TTCCGCCCAT	TCTCCGCCCC	12720
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GGCGCCCGGT	TCTTTTTGTC	AAGACCGACC	TGTCCGGTGC	CCTGAATGAA	CTGCAGGACG	13080
AGGCAGCGCG	GCTATCGTGG	CTGGCCACGA	CGGGCGTTCC	TTGCGCAGCT	GTGCTCGACG	13140
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CTAGATCCTT	TAAATTAATA	AATGAAGTTT	TAAATCAATC	TAAAGTATAT	ATGAGTAAAC	15180
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CTTATTTGAA	CTAACCAATC	AGTTCGCTTC	TCGCTTCTGT	TCGCGCGCTT	CTGCTCCCCG	16620
AGCTCAATAA	AAGAGCCAC	AACCCCTCAC	TCGGGG			16656

## ( 2 ) INFORMATION FOR SEQ ID NO:2:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 24 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATCTCTACGG	TGGTCCTAAA	TAGT	24
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## ( 2 ) INFORMATION FOR SEQ ID NO:3:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 42 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TATATTCTAG	ATTTTTTTTT	TTTTTTTTTT	TTTTTTGAAA	TG	42
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## ( 2 ) INFORMATION FOR SEQ ID NO:4:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 48 base pairs

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( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TATATGGGCC CGATTAGGT GACACTATAG ATTGACGGCG TAGTACAC 4 8

( 2 ) INFORMATION FOR SEQ ID NO:5:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 23 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTGGCAACCG GTAAGTACGA TAC 2 3

( 2 ) INFORMATION FOR SEQ ID NO:6:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 21 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATACTAGCCA CGGCCGGTAT C 2 1

( 2 ) INFORMATION FOR SEQ ID NO:7:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 21 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCCTCTTTTCG ACGTGTCGAG C 2 1

( 2 ) INFORMATION FOR SEQ ID NO:8:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 21 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACCTTGGAGC GCAATGTCCT G 2 1

( 2 ) INFORMATION FOR SEQ ID NO:9:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 21 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCTTTTCAGG GGATCCGCCA C 2 1

( 2 ) INFORMATION FOR SEQ ID NO:10:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 21 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single

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( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTGGCGGATC CCCTGAAAAG G 2 1

( 2 ) INFORMATION FOR SEQ ID NO:11:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGGGCCGTGT GGTCTCATG 2 0

( 2 ) INFORMATION FOR SEQ ID NO:12:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 21 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGGGTCTTCA ACTCACCGGA C 2 1

( 2 ) INFORMATION FOR SEQ ID NO:13:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 22 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CAATTCGACG TAGGCCTCAC TC 2 2

( 2 ) INFORMATION FOR SEQ ID NO:14:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 22 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GAGTGAGGCG TACGTCAAT TG 2 2

( 2 ) INFORMATION FOR SEQ ID NO:15:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 33 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TATATAGATC TAATGAAAGA CCCCACCTGT AGG 3 3

( 2 ) INFORMATION FOR SEQ ID NO:16:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 40 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

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( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TCAATCCCCG AGTGAGGGGT TGTGGGCTCT TTTATTGAGC 40

( 2 ) INFORMATION FOR SEQ ID NO:17:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 36 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCACAACCCC TCACTCGGGG ATTGACGGCG TAGTAC 36

( 2 ) INFORMATION FOR SEQ ID NO:18:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 23 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTGGCAACCG GTAAGTACGA TAC 23

( 2 ) INFORMATION FOR SEQ ID NO:19:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 22 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGTAACAAGA TCTCGTGCCG TG 22

( 2 ) INFORMATION FOR SEQ ID NO:20:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 53 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TATATATATA TGCGGCCGCT TTCTTTTATT AATCAACAAA ATTTTGTTTT TAA 53

( 2 ) INFORMATION FOR SEQ ID NO:21:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 48 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TATATGAGCT CTTTTTTTTT TTTTTTTTTT TTTTTTGAAA TGTTAAAA 48

( 2 ) INFORMATION FOR SEQ ID NO:22:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:22:

-continued

TATATCTCGA GGGTGGTGTT GTAGTATTAG TCAG

3 4

## ( 2 ) INFORMATION FOR SEQ ID NO:23:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 43 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TATATGGGCC CTTAAGACCA TCGGAGCGAT GCTTTATTTTCCC

4 3

## ( 2 ) INFORMATION FOR SEQ ID NO:24:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 18 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TCTCTACGGT GGTCTTAA

1 8

## ( 2 ) INFORMATION FOR SEQ ID NO:25:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 5 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:25:

S e r L e u A r g T r p S e r  
1 5

## ( 2 ) INFORMATION FOR SEQ ID NO:26:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 26 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CATCTCTACG GTGGTCCTAA ATAGTC

2 6

## ( 2 ) INFORMATION FOR SEQ ID NO:27:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TCGAGACTAT TTAGGACCAC CGTAGAGATG GGCC

3 4

## ( 2 ) INFORMATION FOR SEQ ID NO:28:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 25 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CCCTTGATACG GCTAACCTAA AGGAC

2 5



## ( 2 ) INFORMATION FOR SEQ ID NO:29:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 33 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCGAGTCCTT TAGGTTAGCC GTACAAGGGG GCC

3 3

## ( 2 ) INFORMATION FOR SEQ ID NO:30:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 26 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CATCGCTACG GTGGTCCTAA ATAGTC

2 6

## ( 2 ) INFORMATION FOR SEQ ID NO:31:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TCGAGACTAT TTAGGACCAC CGTAGCGATG GGCC

3 4

## ( 2 ) INFORMATION FOR SEQ ID NO:32:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 48 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CGGAAATAAA GCATCTCTAC GGTGGTCCTA AATAGTCAGC ATAGTACC

4 8

## ( 2 ) INFORMATION FOR SEQ ID NO:33:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 56 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TCGAGGTACT ATGCTGACTA TTAGGACCA CCGTAGAGAT GCTTTATTTC CGGGCC

5 6

## ( 2 ) INFORMATION FOR SEQ ID NO:34:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 41 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TATATGCGGC CGCTCTAGAT TACAATTTGG ACTTTCGCC C

4 1

-continued

## ( 2 ) INFORMATION FOR SEQ ID NO:35:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 44 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TATATATGAG CTCTTACAAA TAAAGCAATA GCATCACAAA TTTC

4 4

## ( 2 ) INFORMATION FOR SEQ ID NO:36:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 36 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:36:

TATATGAATT CGTTTGGACA AACCACAACT AGAATG

3 6

## ( 2 ) INFORMATION FOR SEQ ID NO:37:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 44 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TATATATGAG CTCTAATAAA ATGAGGAAAT TGCATCGCAT TGTC

4 4

## ( 2 ) INFORMATION FOR SEQ ID NO:38:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 43 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TATATGAATT CATAGAATGA CACCTACTCA GACAATGCCA TGC

4 3

## ( 2 ) INFORMATION FOR SEQ ID NO:39:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 46 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:39:

TATATGAGCT CGGGTCGGCA TGGCATCTCC ACCTCCTCGC GGTCCG

4 6

## ( 2 ) INFORMATION FOR SEQ ID NO:40:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 52 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:40:

TCCACCTCCT CGCGGTCCGA CCTGGGCATC CGAAGGAGGA CGCACGTCCA CT

5 2

## ( 2 ) INFORMATION FOR SEQ ID NO:41:

-continued

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 48 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TATATGAGCT CCTCCCTTAG CCATCCGAGT GGACGTGCGT CCTCCTTC

4 8

## ( 2 ) INFORMATION FOR SEQ ID NO:42:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 47 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TATATGCGGC CGCTTTCTTT TATTAATCAA CAAAATTTTG TTTTAA

4 7

## ( 2 ) INFORMATION FOR SEQ ID NO:43:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 37 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:43:

TATATGAGCT CGAAATGTTA AAAACAAAAT TTTGTTG

3 7

## ( 2 ) INFORMATION FOR SEQ ID NO:44:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TATATATAGA TCTTTGACAT TGATTATTGA CTAG

3 4

## ( 2 ) INFORMATION FOR SEQ ID NO:45:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 42 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CCGTCAATAC GGTTCATAA ACGAGCTCTG CTTATATAGA CC

4 2

## ( 2 ) INFORMATION FOR SEQ ID NO:46:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 38 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GCTCGTTTAG TGAACCGTAT TGACGGCGTA GTACACAC

3 8

## ( 2 ) INFORMATION FOR SEQ ID NO:47:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 33 base pairs

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( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TATATATAGA TCTGGTGTGG AAAGTCCCCA GGC 3 3

( 2 ) INFORMATION FOR SEQ ID NO:48:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 31 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CTACGCCGTC AATGCCGAGG CGGCCTCGGC C 3 1

( 2 ) INFORMATION FOR SEQ ID NO:49:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 37 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GGCCGCCTCG GCATTGACGG CGTAGTACAC ACTATTG 3 7

( 2 ) INFORMATION FOR SEQ ID NO:50:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 41 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:50:

TATATATCTC GAGAAGCTCT AAGGTAAATA TAAAATTAC C 4 1

( 2 ) INFORMATION FOR SEQ ID NO:51:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 38 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TATATATCTC GAGAGGTTGG AATCTAAAAT ACACAAAC 3 8

( 2 ) INFORMATION FOR SEQ ID NO:52:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 43 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:52:

TATATATGCG GCCGCAAGCT CTAAGGTAAA TATAAAATTT ACC 4 3

( 2 ) INFORMATION FOR SEQ ID NO:53:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 40 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single

-continued

( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:53:

TATATATGCG GCCGCAGGTT GGAATCTAAA ATACACAAAC

4 0

( 2 ) INFORMATION FOR SEQ ID NO:54:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 35 base pairs

( B ) TYPE: nucleic acid

( C ) STRANDEDNESS: single

( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:54:

TCGAGCACGT GCGCGCCTG ATCACGCGTA GGCTT

3 5

( 2 ) INFORMATION FOR SEQ ID NO:55:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 35 base pairs

( B ) TYPE: nucleic acid

( C ) STRANDEDNESS: single

( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CTAGAGGCCT ACGCGTGATC AGGCGCGCCA CGTGC

3 5

( 2 ) INFORMATION FOR SEQ ID NO:56:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 35 base pairs

( B ) TYPE: nucleic acid

( C ) STRANDEDNESS: single

( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:56:

TATATCTCCA GATGAGGTAC ATGATTTTAG GCTTG

3 5

( 2 ) INFORMATION FOR SEQ ID NO:57:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 40 base pairs

( B ) TYPE: nucleic acid

( C ) STRANDEDNESS: single

( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:57:

TATATATCGA TTCAAGGCAT TTTCTTTTCA TCAATAAAAC

4 0

( 2 ) INFORMATION FOR SEQ ID NO:58:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 35 base pairs

( B ) TYPE: nucleic acid

( C ) STRANDEDNESS: single

( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:58:

TATATCTCCA GATGATGACA ATGTGGTGTC TGACG

3 5

( 2 ) INFORMATION FOR SEQ ID NO:59:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 32 base pairs

( B ) TYPE: nucleic acid

( C ) STRANDEDNESS: single

( D ) TOPOLOGY: linear

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( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:59:	
TATATATCGA TTCATGACGA CCGGACCTTG CG	3 2
( 2 ) INFORMATION FOR SEQ ID NO:60:	
( i ) SEQUENCE CHARACTERISTICS:	
( A ) LENGTH: 28 base pairs	
( B ) TYPE: nucleic acid	
( C ) STRANDEDNESS: single	
( D ) TOPOLOGY: linear	
( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:60:	
TATATGGGCC CCCCCCCCCC CCCCACCG	2 8
( 2 ) INFORMATION FOR SEQ ID NO:61:	
( i ) SEQUENCE CHARACTERISTICS:	
( A ) LENGTH: 30 base pairs	
( B ) TYPE: nucleic acid	
( C ) STRANDEDNESS: single	
( D ) TOPOLOGY: linear	
( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:61:	
TATATATCGA TCCCCCCCCC CCCCCAACG	3 0
( 2 ) INFORMATION FOR SEQ ID NO:62:	
( i ) SEQUENCE CHARACTERISTICS:	
( A ) LENGTH: 34 base pairs	
( B ) TYPE: nucleic acid	
( C ) STRANDEDNESS: single	
( D ) TOPOLOGY: linear	
( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:62:	
TATATCCATG GCTTACAATC GTGGTTTTCA AAGG	3 4
( 2 ) INFORMATION FOR SEQ ID NO:63:	
( i ) SEQUENCE CHARACTERISTICS:	
( A ) LENGTH: 33 base pairs	
( B ) TYPE: nucleic acid	
( C ) STRANDEDNESS: single	
( D ) TOPOLOGY: linear	
( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:63:	
TATATGGGCC CTCGATGAGT CTGGACGTTC CTC	3 3
( 2 ) INFORMATION FOR SEQ ID NO:64:	
( i ) SEQUENCE CHARACTERISTICS:	
( A ) LENGTH: 33 base pairs	
( B ) TYPE: nucleic acid	
( C ) STRANDEDNESS: single	
( D ) TOPOLOGY: linear	
( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:64:	
TATATATCGA TTCGATGAGT CTGGACGTTC CTC	3 3
( 2 ) INFORMATION FOR SEQ ID NO:65:	
( i ) SEQUENCE CHARACTERISTICS:	
( A ) LENGTH: 37 base pairs	
( B ) TYPE: nucleic acid	
( C ) STRANDEDNESS: single	
( D ) TOPOLOGY: linear	
( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:65:	

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TATATCCATG GATCCAATTT GCTTTATGAT AACCAATC

3 7

## ( 2 ) INFORMATION FOR SEQ ID NO:66:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 30 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:66:

TATATGGGCC CGGTCGACGC CGGCCAAGAC

3 0

## ( 2 ) INFORMATION FOR SEQ ID NO:67:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 30 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:67:

TATATATCGA TGGTCGACGC CGGCCAAGAC

3 0

## ( 2 ) INFORMATION FOR SEQ ID NO:68:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 32 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:68:

TATATCCATG GTGCCAGCCA GTTGGGCAGC AG

3 2

## ( 2 ) INFORMATION FOR SEQ ID NO:69:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 23 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:69:

TTAATTAACG GCCGCCACCA TGG

2 3

## ( 2 ) INFORMATION FOR SEQ ID NO:70:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 13 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:70:

TAACGGCCGC CAC

1 3

## ( 2 ) INFORMATION FOR SEQ ID NO:71:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:71:

CCATGGTGGC GGCCGTTAAT

2 0

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## ( 2 ) INFORMATION FOR SEQ ID NO:72:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 16 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:72:

GGTTTAAACA GGAGCT

1 6

## ( 2 ) INFORMATION FOR SEQ ID NO:73:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 16 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:73:

CCTGTTTAAA CCAGCT

1 6

## ( 2 ) INFORMATION FOR SEQ ID NO:74:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 47 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:74:

TATATGCGGC CGCACCACCA CCATGAATAG AGGATTCTTT AACATGC

4 7

## ( 2 ) INFORMATION FOR SEQ ID NO:75:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:75:

TATATGCGGC CGCTCATCTT CGTGTGCTAG TCAG

3 4

## ( 2 ) INFORMATION FOR SEQ ID NO:76:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 61 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:76:

TATATGCGGC CGCATCTCTA CGGTGGTCCT AAATAGTACC ACCACCATGA ATAGAGGATT

6 0

C

6 1

## ( 2 ) INFORMATION FOR SEQ ID NO:77:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 25 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:77:

CTCATCGATC AGATCTGACT AGTTG

2 5



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## ( 2 ) INFORMATION FOR SEQ ID NO:78:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 33 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:78:

GATCCAAC TA GTCAGATCTG ATCGATGAGG GCC

3 3

## ( 2 ) INFORMATION FOR SEQ ID NO:79:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 56 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:79:

ACTTATCGAT GGTTC TAGAC TCCCTTAGCC ATCCGAGTGG ACGTGCGTCC TCCTTC

5 6

## ( 2 ) INFORMATION FOR SEQ ID NO:80:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 52 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:80:

TCCACCTCCT CGCGGTCCGA CCTGGGCATC CGAAGGAGGA CGCACGTCCA CT

5 2

## ( 2 ) INFORMATION FOR SEQ ID NO:81:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 57 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:81:

TCGGACCGCG AGGAGGTGGA GATGCCATGC CGACCCATTG ACGGCGTAGT ACACACT

5 7

## ( 2 ) INFORMATION FOR SEQ ID NO:82:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 36 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:82:

CTGGACTAGT TAATACTGGT GCTCGGAAAA CATTCT

3 6

## ( 2 ) INFORMATION FOR SEQ ID NO:83:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 40 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:83:

GTCAAGCTTG CTAGCTACAA CACCACCACC ATGAATAGAG

4 0

## ( 2 ) INFORMATION FOR SEQ ID NO:84:

-continued

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 40 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:84:

CAGTCTCGAG TTACTACCAC TCTTCTGTCC CTTCCGGGGT 40

## ( 2 ) INFORMATION FOR SEQ ID NO:85:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 43 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:85:

TATATGCGGC CGCACCACCA TGTCCGCAGC ACCACTGGTC ACG 43

## ( 2 ) INFORMATION FOR SEQ ID NO:86:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:86:

TATATAGATC TCTTGATCAG CTTCAGAAGA TGGC 34

## ( 2 ) INFORMATION FOR SEQ ID NO:87:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 24 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:87:

TCAATGGCGG GAAGAGGCGG TTGG 24

## ( 2 ) INFORMATION FOR SEQ ID NO:88:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 31 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:88:

CCGCCTCTTC CCGCCATTGA CGGCGTAGTA C 31

## ( 2 ) INFORMATION FOR SEQ ID NO:89:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:89:

TATATAGATC TCTTGATCAG CTTCAGAAGA TGGC 34

## ( 2 ) INFORMATION FOR SEQ ID NO:90:

## ( i ) SEQUENCE CHARACTERISTICS:

-continued

( A ) LENGTH: 44 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:90:

TATATATATG CGGCCGACAC GCCAAGATGT TCCCGTTCCA GCCA

44

( 2 ) INFORMATION FOR SEQ ID NO:91:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 38 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:91:

TATATATATG CGGCCGCTCA ATTATGTTTC TGGTTGGT

38

( 2 ) INFORMATION FOR SEQ ID NO:92:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 35 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:92:

CTCGAGCTCG AGGCACCAGC ACCATGCAAC TTTTT

35

( 2 ) INFORMATION FOR SEQ ID NO:93:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 29 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:93:

CTACTAGATC CCTAGATGCT GGATCTTCC

29

( 2 ) INFORMATION FOR SEQ ID NO:94:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 29 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:94:

GGAAGATCCA GCATCTAGGG ATCTAGTAG

29

( 2 ) INFORMATION FOR SEQ ID NO:95:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 26 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:95:

GGGCGATATC AAGCTTATCG ATACCG

26

( 2 ) INFORMATION FOR SEQ ID NO:96:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 26 base pairs  
 ( B ) TYPE: nucleic acid

-continued

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( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:96:

GGGCGATATC AAGCTTATCG ATACCG 26

( 2 ) INFORMATION FOR SEQ ID NO:97:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 19 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:97:

AATACGACTC ACTATAGGG 19

( 2 ) INFORMATION FOR SEQ ID NO:98:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 29 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:98:

CTACTAGATC CCTAGATGCT GGATCTTCC 29

( 2 ) INFORMATION FOR SEQ ID NO:99:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 17 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:99:

ATTAACCCTC ACTAAAG 17

( 2 ) INFORMATION FOR SEQ ID NO:100:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 29 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:100:

GGAAGATCCA GCATCTAGGG ATCTAGTAG 29

( 2 ) INFORMATION FOR SEQ ID NO:101:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 17 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:101:

ATTAACCCTC ACTAAAG 17

( 2 ) INFORMATION FOR SEQ ID NO:102:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 19 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:102:

AATACGACTC ACTATAGGG

1 9

( 2 ) INFORMATION FOR SEQ ID NO:103:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:103:

CCTCGAGCTC GAGCTTGGGT GGCTTTGGGG CATG

3 4

( 2 ) INFORMATION FOR SEQ ID NO:104:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 17 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:104:

ATTACCCCTC ACTAAAG

1 7

( 2 ) INFORMATION FOR SEQ ID NO:105:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 44 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:105:

CCCTCGAGCT CGAGGGGTCA CTGAGAACT AGAAAAAGAA TTAG

4 4

( 2 ) INFORMATION FOR SEQ ID NO:106:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 37 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:106:

CCGCGGCCGC GTATCTGTGG GAGCCTCAAG GGAGAAC

3 7

( 2 ) INFORMATION FOR SEQ ID NO:107:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 44 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:107:

CGCGCGGGCC CTGTGACATT GAATAGAGTG AGGGTCCTGT TGGG

4 4

( 2 ) INFORMATION FOR SEQ ID NO:108:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 45 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:108:

-continued

AAAGGTTTCA CATTGTAGC TTGCTGTGTC ATTGCGATCT CTACG

4 5

( 2 ) INFORMATION FOR SEQ ID NO:109:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 45 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:109:

GTGGTCCTAA ATAGTTCACT CTATTCAATG TCACACTCGA GCCGG

4 5

( 2 ) INFORMATION FOR SEQ ID NO:110:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 33 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:110:

TATATTCTAG AGCAAGCAAC AGTTACTGCG ACG

3 3

( 2 ) INFORMATION FOR SEQ ID NO:111:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 33 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:111:

TATATATCGA TCCGAAGCGT AGAGTCACAC TTG

3 3

( 2 ) INFORMATION FOR SEQ ID NO:112:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 18 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:112:

TTAACTGTCA AAAGCCAC

1 8

( 2 ) INFORMATION FOR SEQ ID NO:113:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 68 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:113:

CGATGTGGCT TTTAGATGTT AAACCAGAGA AACACACGGA CTTCGGTCCG TGGTATATTA

6 0

GCTGGTAT

6 8

( 2 ) INFORMATION FOR SEQ ID NO:114:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 70 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:114:

-continued

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CTAGATACCA GCTAATATAC CACGGACCGA AGTCCGTGTG TTTCTCTGGT TTAACATCTA 60  
AAAGCCACAT 70

( 2 ) INFORMATION FOR SEQ ID NO:115:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 42 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:115:

TATATCTCGA GACCAACCATG AGTGCTGTAA GTAATAGGAA GC 42

( 2 ) INFORMATION FOR SEQ ID NO:116:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 36 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:116:

TATATCTCGA GCTAGAAGGC AAACCTAACA CCCAAC 36

( 2 ) INFORMATION FOR SEQ ID NO:117:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 31 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:117:

TATATGGGCC CTACATGTCC CACTGTTCAA G 31

( 2 ) INFORMATION FOR SEQ ID NO:118:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 31 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:118:

TATATGGGCC CGTACGGAAG GAAAGAAGTC A 31

( 2 ) INFORMATION FOR SEQ ID NO:119:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 32 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:119:

TATATGGGCC CATTTTGGTT TTGCTATGCG TA 32

( 2 ) INFORMATION FOR SEQ ID NO:120:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 16 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:120:

-continued

TCTCTGTCCT CCATGA

16

## ( 2 ) INFORMATION FOR SEQ ID NO:121:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 66 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:121:

TCGAGTCATG GAGAGAGGAG AACCAGAGAA ACACACGGAC TTCGGTCCGT GGTATATTAC 60

CTGGAT 66

## ( 2 ) INFORMATION FOR SEQ ID NO:122:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 64 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:122:

CGATCCAGGT AATATACCAC GGACCGAAGT CCGTGTGTTT CTCTGGTTCT CCTCTCTCCA 60

TGAC 64

## ( 2 ) INFORMATION FOR SEQ ID NO:123:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 35 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:123:

GCCTCGAGAC AATGTACAGG ATGCAACTCC TGTCT 35

## ( 2 ) INFORMATION FOR SEQ ID NO:124:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 36 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:124:

GAATCGATTT ATCAAGTCAG TGTGGAGAT GATGCT 36

## ( 2 ) INFORMATION FOR SEQ ID NO:125:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 31 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:125:

TATATGGGCC CATCGAGGTG AGAAAGAGGA C 31

## ( 2 ) INFORMATION FOR SEQ ID NO:126:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 31 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear



-continued

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:126:

TATATGGGCC CTGTATCTGG CGGACCCGTG G

3 1

( 2 ) INFORMATION FOR SEQ ID NO:127:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 31 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:127:

TATATGGGCC CGCAGACAAG ACGCGCGGCG C

3 1

( 2 ) INFORMATION FOR SEQ ID NO:128:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 24 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:128:

AUCUCUACGG UGGUCCUAAA UAGU

2 4

## We claim:

1. A method of stimulating in an animal an immune response to an antigen comprising introducing into susceptible target cells a eukaryotic layered vector initiation system comprising a eukaryotic promoter 5' of viral cDNA which initiates within said cell the 5' to 3' synthesis of RNA from said cDNA, wherein said RNA comprises a vector construct which autonomously amplifies in said cell and expresses a heterologous nucleic acid sequence, wherein said heterologous nucleic acid sequence encodes an antigen or modified form thereof which stimulates an immune response within an animal.

2. A method according to claim 1 wherein the target cells are infected in vivo.

3. A method according to claim 1 wherein the expressed antigen elicits an immune response selected from the group consisting of cell-mediated immune response, an HLA class I-restricted immune responses, and an HLA class II-restricted immune response.

4. The method according to claim 1, wherein said promoter is a DNA promoter of RNA synthesis.

5. The method according to claim 1, wherein said vector construct which autonomously amplifies in a cell comprises a sequence which initiates transcription of alphavirus RNA following the 5' promoter, a nucleic acid sequence which encodes alphavirus nonstructural proteins, an alphavirus RNA polymerase recognition sequence, and a 3' polyadenylate tract.

6. The method according to claim 1, further comprising a transcription termination sequence.

7. The method according to claim 1, wherein said vector construct which autonomously amplifies in a cell is derived from a virus selected from the group consisting of poliovirus, rhinovirus, coxsackievirus, rubella, yellow fever, RSV, MoMI.V, and Astrovirus.

8. The method according to claim 1, wherein said vector construct which autonomously amplifies in a cell is derived from a virus selected from the group consisting of tobamoviruses, potyviruses, and bromoviruses.

9. The method according to claim 1, wherein said promoter is selected from the group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40 promoter, CaMV 35S promoter, nopaline synthetase promoter, and the CMV promoter.

10. The method according to claim 1, wherein said heterologous nucleic acid sequence is obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, IIPV, EBV, HIV, FeLV, FIV, Hantavirus, HTLV, HTLV, II, and CMV.

11. The method according to claim 1, wherein said heterologous nucleic acid sequence is obtained from HBV.

12. The method according to claim 1, wherein said heterologous nucleic acid sequence is obtained from HCV.

13. The method according to claim 1, wherein said heterologous nucleic acid sequence is obtained from HSV.

14. A eukaryotic layered vector initiation system, comprising a eukaryotic promoter 5' of viral cDNA which initiates within a susceptible target cell the 5' to 3' synthesis of RNA from said cDNA, wherein said RNA comprises a vector construct which autonomously amplifies in a cell and expresses a heterologous nucleic acid sequence which encodes an antigen or modified form thereof that stimulates an immune response within an animal.

15. A eukaryotic layered vector initiation system according to claim 14 wherein the expressed antigen elicits an immune response selected from the group consisting of a cell-mediated immune response, an HLA class I-restricted immune response, and an HLA class II-restricted immune response.

16. The eukaryotic layered vector initiation system according to claim 14 wherein said promoter is a DNA promoter of RNA synthesis.

17. The eukaryotic layered vector initiation system according to claim 14 wherein said vector construct which autonomously amplifies in a cell comprises a sequence which initiates transcription of alphavirus RNA following the 5' promoter, a nucleic acid sequence which encodes alphavirus nonstructural proteins, an alphavirus RNA polymerase recognition sequence, and a 3' polyadenylate tract.

223

18. The eukaryotic layered vector initiation system according to claim 14 further comprising a transcription termination sequence.

19. The eukaryotic layered vector initiation system according to claim 14 wherein said vector construct which autonomously amplifies in a cell is derived from a virus selected from the group consisting of poliovirus, rhinovirus, coxsackievirus, rubella, yellow fever, RSV, MoMLV, and Astrovirus.

20. The eukaryotic layered vector initiation system according to claim 14 wherein said vector construct which autonomously amplifies in a cell is derived from a virus selected from the group consisting of tobamoviruses, potyviruses, and bromoviruses.

21. The eukaryotic layered vector initiation system according to claim 14 wherein said promoter is selected from the group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40

224

promoter, CaMV 35S promoter, nopaline synthetase promoter, and the CMV promoter.

22. The eukaryotic layered vector initiation system according to claim 14 wherein said heterologous nucleic acid sequence is obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, EBV, HIV, FeLV, FIV, Hantavirus, HTLV I, HTLV II, and CMV.

23. The eukaryotic layered vector initiation system according to claim 14 wherein said heterologous nucleic acid sequence is obtained from HBV.

24. The eukaryotic layered vector initiation system according to claim 14 wherein said heterologous nucleic acid sequence is obtained from HCV.

25. The eukaryotic layered vector initiation system according to claim 14 wherein said heterologous nucleic acid sequence is obtained from HSV.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
CERTIFICATE OF CORRECTION

PATENT NO. : 5,814,482  
DATED : September 29, 1998  
INVENTOR(S) : Thomas W. Dubensky, Jr., et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Claim 3, column 221,

Line 45, "consisting of cell-mediated" should read -- consisting of a cell-mediated --.

Claim 10, column 222,

Line 38, "HTLVI" should read -- HTLV I --.

Line 38, "HTLV, II" should read -- HTLV II --.

Claim 14, column 222,

Line 48, "amplifies ill" should read -- amplifies in --.

Claim 22, column 224,

Line 8, "HTLV II" should read -- HTLV II --.

Signed and Sealed this

Twenty-third Day of October, 2001

Attest:

*Nicholas P. Godici*

Attesting Officer

NICHOLAS P. GODICI  
Acting Director of the United States Patent and Trademark Office



US005843723A

**United States Patent** [19]**Dubensky, Jr. et al.**[11] **Patent Number:** **5,843,723**[45] **Date of Patent:** **Dec. 1, 1998**[54] **ALPHAVIRUS VECTOR CONSTRUCTS**

[75] Inventors: **Thomas W. Dubensky, Jr.**, Rancho Sante Fe; **John M. Polo**, San Diego; **Carlos E. Ibanez**, San Diego; **Stephen M. W. Chang**, San Diego; **Douglas J. Jolly**, Leucadia; **David A. Driver**; **Barbara A. Belli**, both of San Diego, all of Calif.

[73] Assignee: **Chiron Corporation**, Emeryville, Calif.

[21] Appl. No.: **739,167**

[22] Filed: **Oct. 30, 1996**

**Related U.S. Application Data**

[63] Continuation of Ser. No. 404,796, Mar. 20, 1995, which is a continuation-in-part of Ser. No. 376,184, Jan. 20, 1995, abandoned, which is a continuation-in-part of Ser. No. 348,472, Nov. 30, 1994, abandoned, which is a continuation-in-part of Ser. No. 198,450, Feb. 18, 1994, abandoned, which is a continuation-in-part of Ser. No. 122,791, Sep. 15, 1993, abandoned.

[51] Int. Cl.<sup>6</sup> ..... **C12P 21/02**; **C12N 15/63**;  
**C12N 5/10**

[52] U.S. Cl. .... **435/69.3**; **435/320.1**; **435/325**;  
**435/235.1**

[58] Field of Search ..... **435/320.1**, **325**,  
**435/69.3**, **235.1**

[56] **References Cited****U.S. PATENT DOCUMENTS**

5,091,309	2/1992	Schlesinger et al.	435/69.1
5,185,440	2/1993	Davis et al.	536/23.72
5,217,879	6/1993	Huang et al.	435/69.1
5,691,177	11/1997	Guber et al.	435/172.3

**FOREIGN PATENT DOCUMENTS**

WO 92/10578	6/1992	WIPO.
WO 94/17813	8/1994	WIPO.
WO 95/07994	3/1995	WIPO.
WO 95/17525	6/1995	WIPO.
WO 95/27044	10/1995	WIPO.
WO 95/27069	10/1995	WIPO.
WO 95/31565	11/1995	WIPO.
WO 95/32733	12/1995	WIPO.

**OTHER PUBLICATIONS**

Weiss et al., "Establishment and Maintenance of Persistent Infection by Sindbis Virus in BHK Cells," *Journal of Virology* 33(1):463-474, 1980.

Dubuisson and Rice, "Sindbis Virus Attachment: Isolation and Characterization of Mutants with Impaired Binding to Vertebrate Cells," *Journal of Virology* 67(6):3363-3374, 1993.

Shirako and Strauss, "Regulation of Sindbis Virus RNA Replication: Uncleaved P123 and nsP4 Function in Minus-Strand RNA Synthesis, Whereas Cleaved Products from P123 Are Required for Efficient Plus-Strand RNA Synthesis," *Journal of Virology* 68(3):1874-1885, 1994.

Lemm et al., "Mutations Which Alter the Level or Structure of nsP4 Can Affect the Efficiency of Sindbis Virus Replication in a Host-Dependent Manner," *Journal of Virology* 64(6):3001-3011, 1990.

Strauss et al., "Identification of the Active Site Residues in the nsP2 Proteinase of Sindbis Virus," *Virology* 191:932-940, 1992.

Lemm et al., "Polypeptide Requirements for Assembly of Functional Sindbis Virus Replication Complexes: A Model for the Temporal Regulation of Minus- and Plus-Strand RNA Synthesis," *The EMBO Journal* 13(12):2925-2934, 1994.

Frolov and Schlesinger, "Comparison of the Effects of Sindbis Virus and Sindbis Virus Replicons on Host Cell Protein Synthesis and Cytopathogenicity in BHK Cells," *Journal of Virology* 68(3):1721-1727, 1994.

Boyer and Haenni, "Infectious Transcripts and cDNA Clones of RNA Viruses," *Virology* 198:415-426, 1994.

Dubensky, Jr. et al., "Sindbis Virus DNA-Based Expression Vectors: Utility for In Vitro and In Vivo Gene Transfer," *Journal of Virology* 70(1):508-519, 1996.

Herweijer et al., "A Plasmid-Based Self-Amplifying Sindbis Virus Vector," *Human Gene Therapy* 6:1161-1167, 1995.

Kuhn et al., "Infectious RNA Transcripts from Ross River Virus cDNA Clones and the Construction and Characterization of Defined Chimeras with Sindbis Virus," *Virology* 182:430-441, 1991.

Liljeström and Garoff, "A New Generation of Animal Cell Expression Vectors Based on the Semliki Forest Virus Replicon," *Bio/Technology* 9:1356-1361, 1991.

Rice et al., "Production of Infectious RNA Transcripts from Sindbis Virus cDNA Clones: Mapping of Lethal Mutations, Rescue of a Temperature-Sensitive Marker, and In Vitro Mutagenesis To Generate Defined Mutants," *Journal of Virology* 61(12):3809-3819, 1987.

Xiong et al., "Sindbis Virus: An Efficient, Broad Host Range Vector for Gene Expression Animal Cells," *Science* 243:1188-1191, 1989.

Grakoui et al., "A cis-Acting Mutation in the Sindbis Virus Junction Region Which Affects Subgenomic RNA Synthesis," *Journal of Virology* 63(12):5216-5227, 1989.

Hertz and Huang, "Utilization of Heterologous Alphavirus Junction Sequences as Promoters by Sindbis Virus," *Journal of Virology* 66(2):857-864, 1992.

Levis et al., "Promoter for Sindbis Virus RNA-Dependent Subgenomic RNA Transcription," *Journal of Virology* 64(4):1726-1733, 1990.

Polo et al., "Alphavirus mediated delivery of ribozyme therapeutics," *J. Cell. Biochem., Suppl.*, 19A:288, abstract No. A6-413, 1995.

(List continued on next page.)

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[57] **ABSTRACT**

The present invention provides compositions and method, for utilizing recombinant alphavirus vectors.

**47 Claims, 30 Drawing Sheets**

## OTHER PUBLICATIONS

- Schlesinger, S. "Alphaviruses—vectors for the expression of heterologous genes," *Trends in Biotechnology* 11:18–22, 1993.
- Baumann and Schendel, "Interleukin-11 Regulates the Hepatic Expression of the Same Plasma Protein Genes as Interleukin-6," *Journal of Biological Chemistry* 266:20424–20427, 1991.
- Davis et al., "In Vitro Synthesis of Infectious Venezuelan Equine Encephalitis Virus RNA from a cDNA Clone: Analysis of a Viable Deletion Mutant," *Virology* 171:189–204, 1989.
- Huang et al., "RNA Viruses as Gene Expression Vectors," *Virus Genes* 3(1):85–91, 1989.
- Oker-Blom and Summers, "Expression of Sindbis Virus 26S cDNA in *Spodoptera frugiperda* (Sf9) Cells, Using a Baculovirus Expression Vector," *Journal of Virology* 63(3):1256–1264, 1989.
- Racaniello and Baltimore, "Cloned Poliovirus Complementary DNA Is Infectious in Mammalian Cells," *Science* 214:916–919, 1981.
- Rice et al., "Expression of Sindbis Virus Structural Proteins via Recombinant Vaccinia Virus: Synthesis, Processing, and Incorporation into Mature Sindbis Virions," *Journal of Virology* 56(1):227–239, 1985.
- Semler et al., "Production of infectious poliovirus from cloned cDNA is dramatically increased by SV40 transcription and replication signals," *Nucleic Acids Research* 12(12):5123–5141, 1984.
- Wen and Schlesinger, "Regulated expression of Sindbis and vesicular stomatitis virus glycoproteins in *Saccharomyces cerevisiae*," *Proc. Natl. Acad. Sci. USA* 83:3639–3643, 1986.
- Berglund et al., "Semliki Forest Virus Expression System: Production of Conditionally Infectious Recombinant Particles," *Bio/Technology* 11:916–920, 1993.
- Bredenbeek and Rice, "Animal RNA virus expression systems," *Seminars in Virology* 3:297–310, 1992.
- Bredenbeek et al., "Sindbis Virus Expression Vectors: Packaging of RNA Replicons by Using Defective Helper RNAs," *Journal of Virology* 67(11):6439–6446, 1993.
- Davis et al., "Protection Against Influenza in Mice By Vaccination With a Venezuelan Equine Encephalitis Virus Vector Expressing the HA Protein," *J. Cell Biochem. Suppl.* 19A:310, Abstract No. J2–308, 1995.
- Geigenmüller-Gnirke et al., "Complementation between Sindbis viral RNAs produces infectious particles with a bipartite genome," *Proc. Natl. Acad. Sci. USA* 88:3253–3257, 1991.
- Johanning et al., "A sindbis virus mRNA polynucleotide vector achieves prolonged and high level heterologous gene expression in vivo," *Nucleic Acids Research* 23(9):1495–1501, 1995.
- Levis et al., "Deletion Mapping of Sindbis Virus DI RNAs Derived from cDNAs Defines the Sequences Essential for Replication and Packaging," *Cell* 44:137–145, 1986.
- Levis et al., "Engineered defective interfering RNAs of Sindbis virus express bacterial chloramphenicol acetyltransferase in avian cells," *Proc. Natl. Acad. Sci. USA* 84:4811–4815, 1987.
- Liljeström, P., "Alphavirus expression systems," *Current Opinion in Biotechnology* 5:495–500, 1994.
- Olive et al., "A Cell Line That Expresses a Reporter Gene in Response to Infection by Sindbis Virus: A Prototype for Detection of Positive Strand RNA Viruses," *Virology* 198:381–384, 1994.
- Owen and Kuhn, "Identification of a Region in the Sindbis Virus Nucleocapsid Protein That Is Involved in Specificity of RNA Encapsulation," *Journal of Virology* 70(5):2757–2763, 1996.
- Raju and Huang, "Analysis of Sindbis Virus Promoter Recognition In Vivo, Using Novel Vectors with Two Subgenomic mRNA Promoters," *Journal of Virology* 65(5):2501–2510, 1991.
- Rolls et al., "Expression of Additional Genes in a Vector Derived from a Minimal RNA Virus," *Virology* 218:406–411, 1996.
- Rolls et al., "Novel Infectious Particles Generated by Expression of the Vesicular Stomatitis Virus Glycoprotein from a Self-Replicating RNA," *Cell* 79:497–506, 1994.
- Sarver and Stollar, "Sindbis Virus-Induced Cytopathic Effect in Clones of *Aedes albopictus* (Singh) Cells," *Virology* 80:390–400, 1977.
- Strauss and Strauss, "The Alphaviruses: Gene Expression, Replication, and Evolution," *Microbiological Reviews* 58(3):491–562, 1994.
- Strauss et al., "Complete Nucleotide Sequence of the Genomic RNA of Sindbis Virus," *Virology* 133:92–110, 1984.
- Watson et al., "A Mutant CHO-K1 Strain with Resistance to Pseudomonas Exotoxin A and Alphavirus Fails To Cleave Sindbis Virus Glycoprotein PE2," *Journal of Virology*, 65(5):2332–2339, 1991.
- Weiss and Schlesinger, "Recombination between Sindbis Virus RNAs," *Journal of Virology* 65(8):4017–4025, 1991.
- Weiss et al., "Evidence for Specificity in the Encapsulation of Sindbis Virus RNAs," *Journal of Virology* 63:5310–5318, 1989.
- Weiss et al., "Interactions between Sindbis virus RNAs and a 68 amino acid derivative of the viral capsid protein further defines the capsid binding site," *Nucleic Acids Research* 22(5):780–786, 1994.
- Zhou et al., "Generation of cytotoxic and humoral immune responses by nonreplicative recombinant Semliki Forest virus," *Proc. Natl. Acad. Sci. USA* 92:3009–3013, 1995.
- Zhou et al., "Self-replicating Semliki Forest virus RNA as recombinant vaccine," *Vaccine* 12(16):1510–1514, 1994.
- International Search Report, PCT Patent Application No. PCT/US94/10469, May 4, 1995.
- European Search Report, EP Patent Application No. 95115460.8, Nov. 15, 1996.

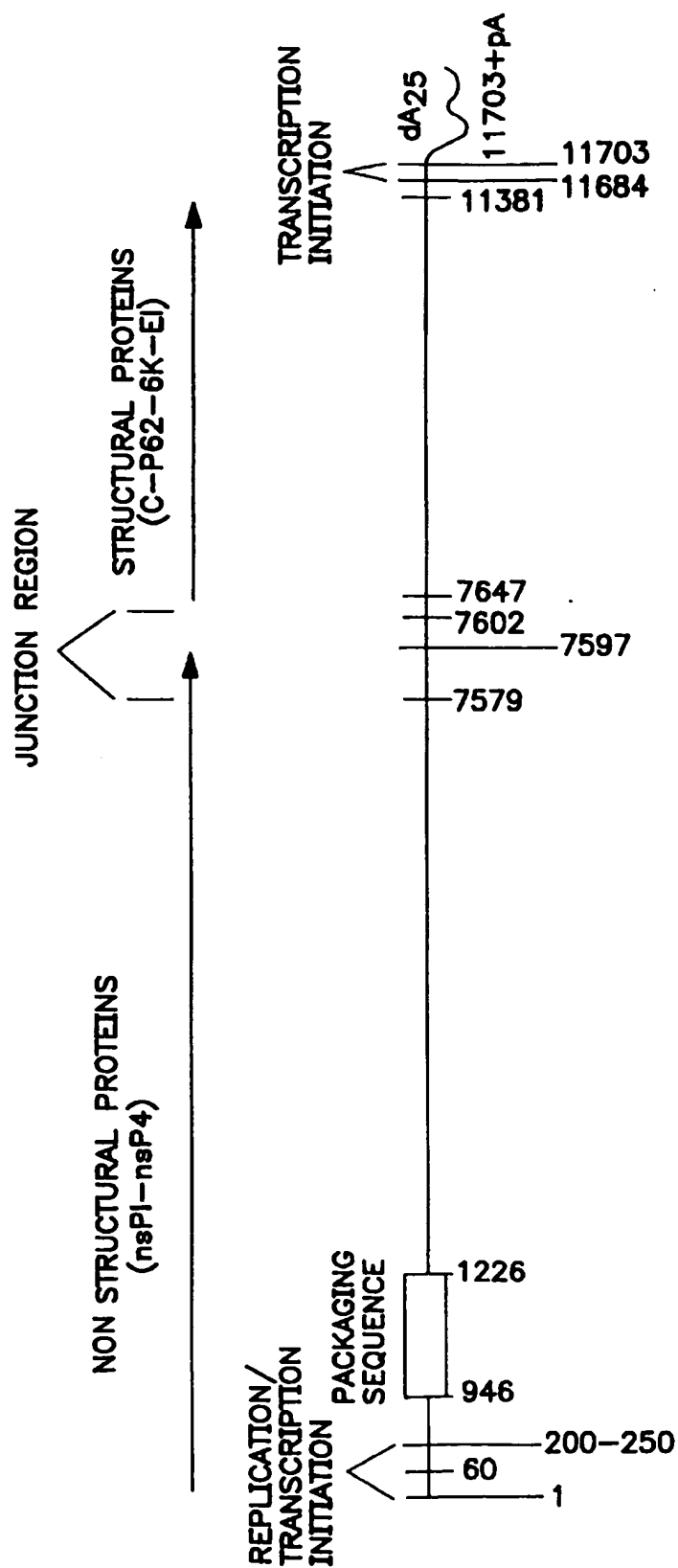


FIG. 1

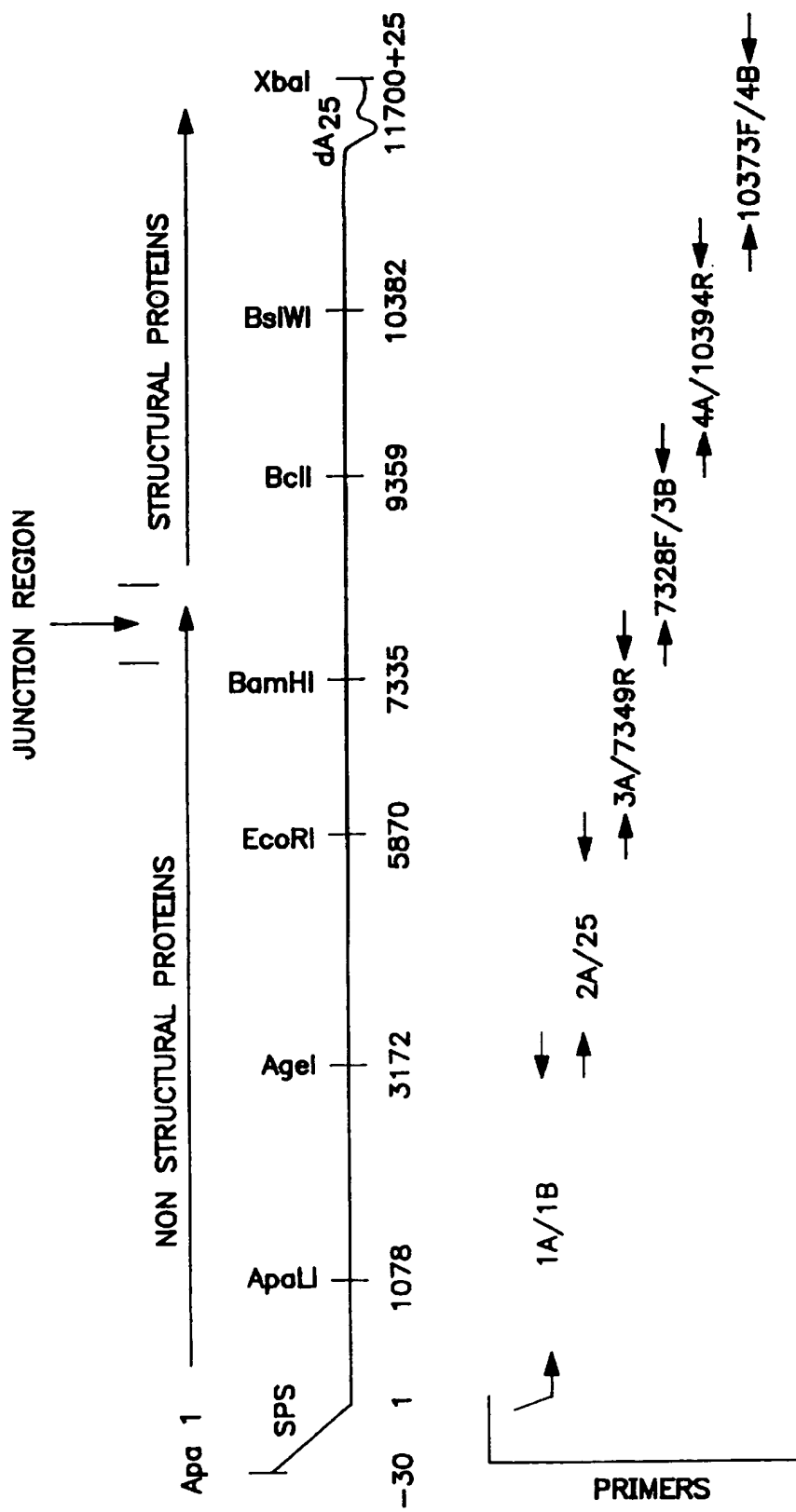


FIG. 2

FIG. 3A

ATTGACGGCG TAGTACACAC  
TGGAGAGGCC AGTAGTAAAC  
AAAAAGCTT CCGCAATTT  
ATGCCAGAGC ATTTTCGCAT  
CGACGATCTT GGACATAGGC  
ATTGTGTCTG CCCCATGCGT  
AACTGGCGGA AAAACGCTGC  
TCCGGACCGT ACTTGATACG  
TTACCTGCAA CATGCGTGCC  
GAACTATCTA TCATCAGGCT  
CCACCCAGTT CATGTTCTCG  
CCGACGAGAA AGTCTTGAA  
GTAGGACAGG AAAATTGTCTG  
ATTCTCCGT AGGATCGACA  
TTCCATCGGT GTTCCACTTG  
TGAGTTGCGA AGGCTACGTA  
CCGTGGGATA CGCGTTTACA  
CAGTAAAGG AGAACGGGTA  
ATCAGATGAC TGGTCTAA TG  
TTGGGCTCAA CCAGCGAATT  
AAAATTACCT TCTGCCGATC  
ATGATCTTGA TAACGAGAAA  
TGTGGCGTT TCGCACTAAG  
GCGTAAAGT CCCAGCCTCT  
TGCCCATGTC GCTGAGGCAG  
TGCTGCAGGT CTCGGAGGAA  
AGGAAGCCAG AGCGGAGAA  
TCGAGGCAGC CGCAGAAATT  
CATTAGTTGA AACCCGCGC  
TCGGACAGTA TATCGTTGTC  
CGACCCCGCT AGCAGATCAG  
CGGTCTGAACC ATACGACGCT  
AATTCCTAGC ACTGAGTGAG  
GCAAACTATA CCACATTGCC  
AGGTTACAAA GGCAGAGCTT  
GCGTTAAGAA GGAAGAAGCC  
ATCATGAGCT AGCTCTGGAG  
CAATAGGAGT GATAGGCACA

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CAATCGCACT  
TCCGTTTGTCT  
CACCTCCAAAT  
GCTGGAGGTT  
GTTTTCCGAG  
CATGATGAAA  
GCATGAGGAG  
GCTCTGCTTT  
CGTGTATATC  
GTACTATATT  
TGCGTACAA  
CAGCACAAAG  
GAAGCCCGGG  
CAGCTTGCAG  
TTGCCGCTGT  
TCCCGGGATC  
GCTATGCAAA  
CATCCCGGCC  
CGATGCACAA  
CAGGAACACC  
ATGGGCTAAG  
CAAGCTTACG  
CCCACCTGGA  
GTCCGTATGG  
ACCAAGAAAG  
TGCTTTTGAG  
ATTAGTGGCA  
CCAGGCGGAC  
TCAAGCAAA  
GAATGCCAAA  
CGGAAGATCA  
AGGTGCCGTA  
CGAAAGAGAG  
TACAGAAGAG  
TGACGTGGAC  
AGAACTGACC  
GGTCCCCTAC  
TATTATCAAG

AACAGCCGAG  
ACCCCCAGAG  
CACAGCAGGT  
AACTAATCGA  
CTCGTAGAAT  
ACCCGGACCG  
ACAAGAACCT  
AACACCATC  
TCATGCAGGA  
TGCGGACCT  
GTTCTACCC  
TCGGACTTTG  
AGAAGGAGTT  
AACACAGAGC  
AGTCGTACAC  
TCACCATCAG  
AGGCTTCTT  
TGTGCACGTA  
TATCACCTGA  
GTAGGACTAA  
GGTTCAGCAA  
CTAGAGAACG  
CGTTTTATCG  
TCCCATGTC  
TGGCATTGCA  
AGGCCAAGGC  
CACTTCCACC  
TGAGGGGCT  
GGATAATACC  
CTGTGCTGAA  
TAACACATC  
TGCCAGCAGG  
TAGTGACAA  
CGCCAAAGAA  
CCGCAAGAA  
AGTACGTGTT  
TCCTCTCGGG  
CCGACCTGCT  
GCAAGTCCCG

TATTGAATCA  
GTAGACGTAG  
GAGGTAGTAG  
CTGGCCAGTA  
AGCGCACCGG  
AGTCCAGAAG  
AAGATTACAA  
CCGGATGCTG  
GAAATTTCCG  
ATGAAAGGCG  
GCTATGGCAG  
GCGGTAAACA  
ATAATGAGGA  
CTTTATCCAG  
AATGGAAGC  
GTGAAAGAAA  
CACAAATAGC  
TCGTTCCCTG  
GCCACGGATA  
GTCAATTAAAG  
ATAGCACAAAG  
ATGCTGGGTA  
AAAGTACATT  
TTTAGCGCTT  
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TTAGTCAATGG  
CTCCGAGAAG  
GTCTGCGAAG  
GGTCACGTAA  
TCGCCAACT  
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AAAGTACTGA  
AGCGCCACGT  
ATGCATGGCC  
GCAGAAACAG  
TCAGGCTCTG  
GGACTGAAGA  
CCGGGGTCTGG

ACCATCACAA  
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GACCATGCTA  
CCTACCCACAG  
CACCAGTATC  
TATGCCAGTA  
ATTAAAGGATC  
CACAAACGATG  
AACGCTCCCG  
GGCTTCGACA  
ACCAACTGGG  
CTGAGTGAAG  
TCGCGGGTTT  
AGCTGGCATC  
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ACGGGAGAAA  
GTTACTGACA  
ACCATTCGCG  
AACTTCGCG  
AACACCATGC  
GAGCGCAAGG  
TATGGCTGCT  
ACGCAGACCT  
ACGACCTCTT  
GATGAAAAC  
GATGCTCAGG  
GACAAAGGCA  
ATCGGAGCAG  
GACCGTATGA  
CTCGCACCCAG  
CTCGCACCCAG  
GGAAGGTACG  
CCATGGCCAG  
TTTGTGAACC  
GGGCAAGTACA  
AAGCAAGCGTT  
AACCTTCCCT  
AAGGTCTGAAA  
TCAACTGTCTA



2340	GAGGCCGACG	TCCGGAATTT	AAGAAAATTG	AGCGGAAAGA	AGCGGATACG	TCTTGTATCC	CGGCACGAGA	2400	ATGCTCAACG	AGATTCGGTT	CGAAGACAGT	CAGATTACGT	GAGGGGTATG	TCTTGTATCC	CGGCACGAGA	2460	CACGCAGGAG	GTTTCGGTGC	TTGACGAAGC	GTGCTGTACG	CAGATTACGT	GAGGGGTATG	AGCGGATACG	CGGCACGAGA	2520	TGCGGAGACC	GGTAGTACTA	CCCGAAGGT	ATCGTCAGGC	ATCGTCAGGC	CGGCACGAGA	2580	CACCTTGAAA	GCATTTCAAT	ACATAAAGG	AACATGATGC	AACATGATGC	CGGCACGAGA	2640	CAGCCAGTTA	CCGTTGCACA	ATATCTCCG	TTCTACAAGT	TTCTACAAGT	CGGCACGAGA	2700	CCGTGCAAGA	AACCAACGAAC	GAAAAGTGA	CATTACGATG	CATTACGATG	CGGCACGAGA	2760	ATCATCTCTGA	GCCAGGGGAT	CAAGCCCGAA	ACAGGGGCCA	ACAGGGGCCA	CGGCACGAGA	2820	GAAGTAATGA	TCCCGGACAT	AAATCGACTA	AAGCAATTGC	AAGCAATTGC	CGGCACGAGA	2880	CAGAAAAGTCA	TGCCGTCCGG	AAGGAGTGA	CTAACCCAGAA	CTAACCCAGAA	CGGCACGAGA	2940	ACCCGCACGT	CGTGTGCTC	AGCATGTGAA	ATCACATCAG	ATCACATCAG	CGGCACGAGA	3000	CTCAGTAACA	GATTAAAGCAG	GCGACCCATG	ACCTTGCAGG	ACCTTGCAGG	CGGCACGAGA	3060	AAGGGAATAA	AGCTGAACAC	AGGACTGGGA	GCTACTATAG	GCTACTATAG	CGGCACGAGA	3120	ACCAACGTTT	CAGCTGCAAG	CCAATCCGTT	ACTCCCCTGT	ACTCCCCTGT	CGGCACGAGA	3180	ACCGGTTGCC	TATCGTACTT	CCACGGCCGG	CCGATACTAG	CCGATACTAG	CGGCACGAGA	3240	ATTTACGCCT	ACATTCGGCC	ATGACAAACC	CAGTTTGC	CAGTTTGC	CGGCACGAGA	3300	TTTTCTAAAC	AAGCGGACTG	TGGACTTGAC	TTTTTCGGCA	TTTTTCGGCA	CGGCACGAGA	3360	CATTTGGACA	GCCGGTAGCT	ATTCAGCGAG	TATCCCGCCG	TATCCCGCCG	CGGCACGAGA	3420	CTCTCCCGTA	TGATTTGCAG	GCCACAACT	GCTGGGAAGG	GCTGGGAAGG	CGGCACGAGA	3480	ACGGGAGGAA	CGGTAATCTT	TCCCGGTGAA	CATAACCTGG	CATAACCTGG	CGGCACGAGA	3540	CCTCACGCCT	CGCAATCTT	GCCCGTTCGA	AAGCAACCCG	AAGCAACCCG	CGGCACGAGA	3600	AACCAGTTCA	AAAATCTTTG	AAATAATTGA	GTATCAGAGG	GTATCAGAGG	CGGCACGAGA	3660	AAGAGAAATCG	AGCTCCCCTG	AGATAAAGAA	ATAGCCGGTG	ATAGCCGGTG	CGGCACGAGA	3720	GCTTTTCGGT	CTACAACCTG	CAGATAAGAA	GACCTGGTGT	GACCTGGTGT	CGGCACGAGA	3780	TACAGAAACC	TGGAACATAA	TCATCAACAT	GACCTGGTGT	GACCTGGTGT	CGGCACGAGA	3840	CGTTCCGGCC	AGCCCTTTCC	CGACCTTAAA	GACCATGCGG	GACCATGCGG	CGGCACGAGA	3900	GCCGACCCGCA	CTATGGCTAC	TGGTGAAGTC	GGCACCTCTG	GGCACCTCTG	CGGCACGAGA	3960	GCCAGCGAGAC	CAGGGTGCTT	GAAAGTTTGT	GCCTCTGCCA	GCCTCTGCCA	CGGCACGAGA	4020	GACAACAGCC	CCGACAACCT	ACCTGATTTT	ACAGAAATGT	ACAGAAATGT	CGGCACGAGA	4080	TATGAGGGTA	TTCGTCCGTG	ATTGCGTGAT	CACCATCTGA	CACCATCTGA	CGGCACGAGA	4140	ATTGCTGACT	AAGGGAGAA	ACCGCACCAA	GCGCCGTCT	GCGCCGTCT	CGGCACGAGA	4200	GAGGGAGTCT	TAGACCAAGC	ATCCGCTGGG	AACGCAGCCA	AACGCAGCCA	CGGCACGAGA	4260	GAGACAGGCA	TTCAGCCACG	GIITTACCGA	TGGCCGACCA	TGGCCGACCA	CGGCACGAGA	4320	CCTGATTTCC	GCGGCTCGGC	AAGTGATCCA	CTAGGAAAGT	CTAGGAAAGT	CGGCACGAGA	4380	GCACTGCGAC	GCCCTACCAT	TGCTACAAAA	CCTTTGAAAT	CCTTTGAAAT	CGGCACGAGA	4440	ACAGGCAAT	ACTGCTATCT	TGCGCAATCC	ATCAAGCTG	ATCAAGCTG	CGGCACGAGA	4500	GCCTAGACA	CTTGACAACC	CACTTAACTG	CTTGAAGTAT	CTTGAAGTAT	CGGCACGAGA	4560	AGAAATCGAC	GTGGAAGGAA	TGGATAAGAA	ATCTATTGCC	ATCTATTGCC	CGGCACGAGA
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**FIG. 3B**

4620	GAGATCGACG	TGAAGATATG	AGCTGAAGGA	TCTGTAACAG	ACTTAAGGAG	CGGCACCTCCA
4680	TTCAGTACTA	AAGAAAGGGA	GCCTTGAAGG	CCAGACAGTT	ATGGATCCAT	ATGATGAGTTAGT
4740	GCAAAAGACA	CCATCAAGCA	GCACCAAAAT	TACTTCGAAG	ATTGTATTCG	ATTGATCAAGGAAA
4800	CTGTGTGCCT	TAATGAACAA	ACCAGGAAAG	TTCCTTAATG	AAAGGTCCTG	AAAGGTCCTG
4860	CATAAAACCGT	CCGGTTCGAC	GGGAAAGTGT	GAGCAATCC	TGAGCAACATG	CCGACCAATG
4920	CCAGAAAAGG	TGCCATGACG	TTTGCATGTA	TGCGGTGCC	GCCCAAAACG	CCGCAAAACG
4980	ACCCCTTC	ATGCTCTCC	AAGTTACAGT	AACGTCAAG	TAGAAGCAAT	TAGAAGCAAT
5040	CTGTTAATC	GAAAGTAGTC	TTCAGTGCAC	GTTCAGAAGG	AAATTAAGAA	AAATTAAGAA
5100	CAGCCTACCG	AGTGCCAGAA	AGTACATAGA	CCCGCCCGTA	CGCATTCGTT	CGCATTCGTT
5160	CCATCTACAG	GACACCGTCA	AAGTTGTAGC	GAGGCCCCCG	ACAGGCCGAG	ACAGGCCGAG
5220	AGTAGCGAAG	TATGGATGAC	TCTCACTGGA	GTCACAGACA	CTCGCTGAT	CTCGCTGAT
5280	GACAGTTGGT	TACTAGTATG	ACCACTCTAT	AGCGATCGG	TTCGAGCTTA	TTCGAGCTTA
5340	GCTGACGTTT	GGTGGTGGTG	CCGAAAGGCT	GAGATAGTAG	TAGTTCACCTA	TAGTTCACCTA
5400	GCCCGCCTGG	AAAGAAGATG	C6CCAAGGCT	CCTATTCCAC	TGAGCCTGCC	TGAGCCTGCC
5460	CTCCACCTCT	CTCTGAGTCC	CAAGCAATAG	ACTCCACCGG	AAAGAGCCCT	AAAGAGCCCT
5520	GCCCGCCAGG	CGGAGAGACG	CAATTTTCGA	TCCCTCGGAT	GGTATCCATG	GGTATCCATG
5580	GGATCGTTTT	TATGTCCTTC	CGGATGTGCC	ACAGGCCCCA	ACCCCTGGCA	ACCCCTGGCA
5640	GTCTTGTTTT	GTCCGAAACC	GAGCAACTGA	CTGAGCCGCA	GATTGATGAG	GATTGATGAG
5700	GTATCTTTTT	CCGATCAGCC	TTATATCGTC	GTGAACCTCA	ACCGGGCGAA	ACCGGGCGAA
5760	CTAACCCGGG	TGAATACTGA	CGAGGAGGAC	AGACGCAGGA	GCAGAGACGT	GCAGAGACGT
5820	AATGTCGGT	CTTGCAAAAG	GCCCTGGGCA	ACGGACACAG	CATATTTTCG	CATATTTTCG
5880	ATTTCATGCC	CCTGGAAAAG	AGCGCAATGT	CCGACCTTGG	GCTTACAGAA	GCTTACAGAA
5940	ATGCCCAACG	GTACCAAGAT	TCAAACCTCAG	GAGGAACAAC	CACGTCGAAA	CACGTCGAAA
6000	ATAACCACTG	TCAGAAAGCC	AAGTAGAAA	CAGTCTCGTA	AAGTAGGTAC	AAGTAGGTAC
6060	GAATGCTATA	AGATCAGCCA	ACTCTGCCAC	CGACTGTATA	GTCAGGACTA	GTCAGGACTA
6120	TCCGATCCAC	GGCGAACTAC	GTACGCTACC	TTGTACTCCA	TCCGAAACCA	TCCGAAACCA
6180	GTAGCATCTT	CTATCCGACA	TGCTAGAGAA	AACAACATCT	AGCTGCTGT	AGCTGCTGT
6240	GTGCGCTGCC	AGACGGGACA	TGGATATGGT	GATGCTTACT	TGCGAGTAC	TGCGAGTAC
6300	CATGAGTATA	CCGAAAAAA	TTAGAAGTTA	CCCGCTAAGC	AACCTTCTGC	AACCTTCTGC
6360	CAAAATGTGC	GAAACACGCTA	CAGCGATGCA	GCGGTTCCAT	TATCCGCGAGT	TATCCGCGAGT
6420	CCAACACTGG	GCGTGAACTG	TCACGCGAGT	AAATGCAACG	AACTAAAAGA	AACTAAAAGA
6480	GAGTATGGG	ATGTAATGAC	GAAAAATATG	GAAATGCTTTC	ATTCAAATGTC	ATTCAAATGTC
6540	TATGTAGCTA	TGTCACCGCA	CCACTGAGTT	ATTAGGATTA	TCGGAAGCCA	TCGGAAGCCA
6600	GTCCCATTCG	GTATAATTIG	TTGCAAAAGAC	GCCACATAT	CCCTAAGGCC	CCCTAAGGCC
6660	GTTCAACCCAG	AGACGCTGAA	ACATGAAAAG	TTCGTCAATG	TATGGATAGA	TATGGATAGA
6720	GTAACCCCTGG	ACAAGCGGCA	TACAAGTAT	AGACCGGAAAG	CACAGAAGAA	CACAGAAGAA
6780	GCCGCTTTCG	TAGGCTTACG	AAATTAGTGG	ATTACCCGGG	CTTATGCGGG	CTTATGCGGG
6840	ATCATAGCAG	TTTTGATGCA	CGCGGGAAGGA	TTTGACATGT	TACACACGCTT	TACACACGCTT

FIG. 3D

AACACTTCAA  
AAGACGACGC  
CACTACTCGA  
GTACTCGTTT  
ACACAGTTTT  
GATGTGCAGC  
TGGCTGAGAG  
GTGAGAGACC  
CGTGCCGCGT  
ACGACGAGCA  
GAGTAGGTAT  
TTACACCTGT  
TCAGAGGGGA  
CTGACTAATA  
GCCCCTTCCC  
TGCCTGCCCG  
TAGTCA TTGG  
AGAAGCAGGC  
AGAAGCAACC  
CCGACAGATT  
TGGAGGAA  
CAAAGCTCAA  
ACATGAGAAG  
ACCACGGAGC  
GAGGAGACAG  
GTGGCGCTGA  
AGACAATTAA  
CAATGTGTTT  
GCGAACCTTC  
CCCTGCTCAA  
ACGACTTTAC  
CGTGCTTCAG  
GCATACAGAC  
ACCGCTACAT  
AGATTAGCAC  
AATGCCCTCC  
GTACACTGGC  
CCGTTACCGG

GCAAGGGCGAC  
TATGGCGTTA  
CTTGATCGAG  
TAAATTCGGG  
GAATGTGTT  
GTTCA TTGGC  
GTGCGCCACC  
ACCTTACTTC  
GGCGGATCCC  
AGACGAAGAC  
AACAGGCAC  
CCTACTGGCA  
AATAAAGCAT  
CTACAAACC  
GGCCCCCACT  
CAACGGGCTG  
ACAGGCAACT  
GCCCAAGCAA  
TGCAAAACCC  
GTTGACGTC  
GGTAATGAAA  
ATTTACCAAG  
TGAGGCATT  
GGTGCAGTAT  
CGGTGCTCCG  
TGAAGGAACA  
GACGACCCCG  
GCTCGGAAAT  
CAGAGCCCTC  
TGCCATA TTG  
CCCTGACCAGC  
CCCTGTTAAG  
TCCCGCCAG  
GTCGCTTAAG  
CTCAGGACCG  
AGGGGACAGC  
CCGCAAGATA  
TAAAGGAATT

CCGGTACTGG  
ACCGGTCTGA  
TGCGCCTTTG  
GCGATGATGA  
ATCGCCAGCA  
GACGACAACA  
TGGCTCAACA  
TGCGGCGGAT  
CTGAAAAGGC  
AGAAGACGCG  
TTAGCAGTGG  
TTGAGAACTT  
CTCTACGGTG  
ACCAACCATGA  
GCCATGTGGA  
GCTTCTCAAA  
AGACCTCAAC  
CCACCGAAGC  
AAACCCGGAA  
AAGAACGAGG  
CCTCTGCACG  
TCGTACGAT  
ACCTACACCA  
AGTGGAGTTA  
ATCATGGATA  
CGAACTGCCC  
GAAGGACAG  
GTGAGCTTCC  
GACATCCTTG  
CGGTGCGGAT  
CCCTACTTGG  
ATCGAGCAGG  
TTTGATACG  
CAGGATCACA  
TGTAAGAGC  
GTAACGGTTA  
AAACCAAAAT  
CCTTGACACAG

AGACGGGATAT  
TGATCTTGGGA  
GAGAAATATC  
AATCCGGAAT  
GAGTACTAGA  
TCAATACATGG  
TGGAGGTTAA  
TTATCTTGCA  
TGTTTAAGTT  
CTCTGCTAGA  
CCGTGACGAC  
TTGCCACAGAG  
GTCCCTAAATA  
ATAGAGGATT  
GGCCGCGGAG  
TCCAGCAACT  
CCCCACGTCC  
CGAAGAAACC  
AGAGACAGCG  
ACGGAGATGT  
TGAAGGAAAC  
ACGACATGGA  
GTGAACACCC  
GATTTACCAT  
ACITCCGGTCCG  
TTTCGGTCTGT  
AAGAGTGGTC  
CATGCGACCG  
AAGAGAACGT  
CGTCTGGCAG  
GCACATGCTC  
TCTGGGACCG  
ACCAAAGCCG  
CCGTTAAAGA  
TTAGCTACAA  
GCATAGTGAG  
TCGTGGGACG  
TGTACGACCG

CGCATCATTC  
GGACCTGGGT  
ATCCACCCAT  
GTTCTCTACA  
AGAGCGGCTT  
AGTAGTATCT  
GATCATCGAG  
AGATTGCGTT  
GGGTAAACCG  
TGAAACAAAG  
CCGGTATGAG  
CAAAAGAGCA  
GTACGATAG  
CTTTAACATG  
AAGGAGGCAG  
GACCAACAGCC  
CATGGCACCT  
CATCGGGCAC  
CATCGACCCAC  
GTTGCGACAG  
CGAAGGATT  
CCCTCGCGGA  
GGTGTGCGG  
CACCTGGAAT  
CGCAGCACCA  
CCCGCCACCA  
GAACCATGAG  
AAGCAAAAGA  
GTACTGCCAT  
AGCGGACGAT  
AGCAGCAAGC  
AGGCACCATG  
AGGATACCTT  
TAGCAACTCA  
GGAAAAATAT  
TCTGAAAAACA

GACAAAAGCC  
GTGGATCAAC  
CTACCTACGG  
CTTTTGTCA  
AAAACGTCCA  
GACAAAGAAA  
GCAGTACAG  
CTCTCCACAG  
CTCCAGCCG  
GCGTGGTTA  
GTAGACAATA  
TTCCAAGCCA  
TACATTTTCA  
CTGGCCGCGC  
GCGGCCCGA  
GAGAAGAAGA  
AAGTTGGAGG  
GCACCTGGCCA  
CCTGTGCTAT  
TTGCCAGTCA  
TATAACTGGC  
GTAGGAGGCA  
ATAGTCTTCG  
AGTAAAGGGA  
CTGGTCACGG  
TGCTATACCC  
GCCCTACGATA  
AGCGTCTGTG  
CATACTGAAC  
AACACCATAC  
GCAAAACAAGT  
GATGACATCA  
CTCTCTGCAA  
GCAACGTCTC  
GATCTACCTC  
ACTGACGGCT

FIG. 3E

ACATCACIAT  
GGAAGTTTA  
ACTACAAGAC  
AGTGCCTCGC  
GACATGACGA  
CCTGCATGGT  
TCCAATTAGA  
AACCACACAC  
GCCGGAATA  
CAGGAGACCC  
TGACACCAT  
CAGTGTATG  
ACGCCGTAA  
CGTACACCGA  
TGTCATACC  
TTTGTAGTGT  
TTCCAAATGT  
TCAATTTGGA  
TTACCTGCAA  
AATGTCAGCC  
TTATGTGGGG  
ACGTCGAAT  
CCGCGATGAA  
ACGTGAACGG  
CAGCATCGTT  
ATGACTTCCC  
CCTTGACTAG  
AGAACGTGCA  
CAGGCCGCCC  
GAGCGGTGGA  
TTATCAGGAC  
CTTATTCAGC  
AATGCCCCGT  
TGGAGAAAGG  
TATCGCTGTG  
TCGTGAGCAC  
GGAGTTGGCT  
TTTTTTGCTTG

GCACAGGGCCG  
CGCAAGCCCG  
CGGAACCGTT  
CTATAAGAGC  
CCACACGGCC  
CCCTGTTGCC  
TACAGACCCAC  
TGAATGGATC  
CATAAGGGA  
TCACGGATGG  
CTTAGCCGTC  
TGCCGTGAAA  
CCCAACTTCG  
GACCATGAGT  
TTTGCCGCGG  
TGCCGGCGCC  
GCCACAGATA  
GATCACGTGC  
ATTACCCACT  
GGCCGCTCAT  
AGGAGCGCAA  
GTACGAGAT  
AGTAGGACTG  
AGTCACACCA  
TACGCCATTG  
GGAATATGGA  
CAAGGATCTC  
TGTCGCCGTAC  
ACTGCAGGAA  
CTGTTTCATAC  
ATCAGATGCA  
AGACTTCGGC  
ACATTCGCAT  
AGCGGTGACA  
TGGGAAAGAA  
CCGACACAA  
GTTTGCCCTT  
CAGCATGATG

GGACCGCAGCG  
CCATCTGGGA  
TCGACCCGCA  
GACCAACGGA  
CAAGGGAAT  
TACGACATTGC  
GTGGAATGGA  
GTCGGAAGCA  
AATCATGAGC  
CCACACGAAA  
GCATCAGCTA  
GCGCGCCGTG  
CTGGCACTCT  
TACTTGTGGT  
TTTATCGTTC  
TACCTGGCGA  
CCGTATAAGG  
ATGTCTTCGG  
GTGGTCCCCT  
GCAGACTATA  
TGTTTTTGCG  
TGCGGCTCTG  
CGTATAGTGT  
GGAACGTCTA  
GATCATTAAGG  
GCGATGAAC  
ATCGCCAGCA  
ACGCGAGCCG  
ACCGCACCTT  
GGGAACATTC  
CCACTGGCT  
GGGATGGCCA  
TCGAGCACAG  
GTACACTTTA  
ACAACATGCA  
AATGACCAAG  
TTGCGCGGGG  
CTGACTAGCA

CTTATACATC  
AGAACAATTAC  
CCGAAATCAC  
AGTGGGICTT  
TGCAATTTGCC  
ATGTAATACA  
TCACCACCCAG  
CAGTCAGAAA  
CGGTGAGGGT  
TAGTACAGCA  
CCGTGGCGAT  
AGTGCCTGAC  
TGTCCTGCGT  
CGAACAGTCA  
TAATGCGCTA  
AGGTAGACGC  
CAGTTGTTGA  
AGTTTTTGCC  
CCCCAAAAT  
CCTGCAAGGT  
ACAGTGAGAA  
ACCACGCGCA  
ACGGGAACAC  
AAGACTTGAA  
TCGTTATCCA  
CAGGAGCGTT  
CAGACATTAG  
CATCAGGATT  
TCGGGTGTAA  
CCATTTCTAT  
CAACAGTCAA  
CCCTGCAGTA  
GCACCTCCCA  
GCACCGCGAG  
ATGCAGAAATG  
AATTTCAAGC  
CCTCGTCGCT  
CACGAAAGATG

CTACCTGGAA  
GTATGAGTGC  
TGTTGACACC  
CAACTCACCG  
TTTCAAGTTG  
TGGCTTTAAA  
GAGACTAAGG  
CTTACCCGTC  
CTATGCCCAA  
TTACTACCAT  
GATGATTGGC  
GCCATACGCC  
TAGGTCGGCC  
GCCGTTCTTC  
CTGCTCCTGC  
CTACGAACAT  
AAGGCGAGGG  
TTCCACCAAC  
CAAAATGCTGC  
CTTCGGAGGG  
CAGCCAGATG  
GCGGATTAA  
TACCAATTTT  
AGTCATAGCT  
TCGCGGCCCTG  
CGGAGACATT  
GCTACTCAAG  
TGAGATGTGG  
GATTGCAGTA  
TGACATCCCG  
ATGTGAAGTC  
TGTATCCGAC  
AGAGTCGACA  
TCCACAGGGG  
TAAACCAACCA  
CGCCATCTCA  
ATTAATTATA  
ACCGCTACGC

GAATCATCAG  
AAGTCGGCG  
GCCATCAAGC  
GACTTGATCA  
ATCCCGGGTG  
CACATCAGCC  
GCAAAACCCGG  
GACCGAGATG  
GAGTCAGCAC  
CGCCATCCTG  
GTAACCTGTTG  
CTGGCCCCAA  
AATGCTGAAA  
TGGGTCCAGT  
TGCTGCTT  
GCGACCACTG  
TATGCCCCGC  
CAAGAGTACA  
GGCTCCTTGG  
GTCTACCCCT  
AGTGAGGCGT  
GTGCACTACTG  
CTAGATGTGT  
GGACCAATTT  
GTGTACAAT  
CAAGCTACCT  
CCTTCGCCA  
AAAACCAACT  
AATCCGCTCC  
AACGCTGCC  
AGTGAGTGCA  
CGCGAAGGTC  
GTACATGTCC  
GACTTTATCG  
GCTGACCTA  
AAACATCAT  
GGAATATGA  
CCCAATGATC

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FIG. 3F

CGACCAGCAA AACTCGAIGT CCGCTTACCG TGCAATAATGC CCAAAACTC ACTTTTATTA AAAAAAAA CTGATCAGCC GCTTCCCTTG GGCATCGCAT CAAGGGGGAG TTCGAGGGC CGATTAAAGC CCTAGCGCCC CCGTCAAGCT CGACCCCAA GGTTTTTCGC TGGAAACAAC TTCGGCTTAT TCGGCTTAT TGGAACTGTG GCAAGCATG GCAAGCATG AGGCAGAAGT AGGCAGAAGT TCCGCCCATC ATGGCTGACT AATTTTTTTT TCCAGAGGCA GTGAGGAGGC CTTGTATATC CATTTCGGA AACAAGATGG ATTGCACGCA ACTGGGCACA GCGGCCCGGT TCTTTTGTG GCTATCGTGG AGGCAGCGCG TTGTCACCTGA AGCGGGAAGG CATTGCTCCT TGATCCGGCT TGATCCGGCT TCGGATGGAA GCCAGCCGAA AGGGCTCCTG GACCCATGGC CATCGACTGT TTTCTGGATT TGGCTACCCG TTTACGGTAT

ACTTCCGAGG CCGGCAATAT TGCAGGTGT AATGTAATTC TTTCTTTTAT AAAAATCTAG TCGACTGTGC ACCCTGGAAG TGCTGAGTA GATTGGGAG GAAAGAACCA GCGGCGGGTG GCTCCTTTTCG CTAATTCGGG AAACCTGATT CCTTTGACGT CTCAACCCCTA TGGTTAAAA GTGAGTTAGG GTCACTCAAT CATCTCAAT ATGCAAGCA CCGCCCTTAA ATTTATGCAG TTTTTCGGAG TCTGATCAAG GGTCTCCGG GGTCTCCGG GGTCTCCGG AAGACCGACC CTGGCCACGA GACTGGCTGC GCCGAGAAAG ACCTGCCCAT GCGGCTCTTG GCGGCTCTTG TCGATCAGGA GGCTCAAGGC TGCCGAATAT GTGTGGCGGA GCGGCGGAATG GCATCGGCTT

AACIGATGIG AGCAACACTA TGCCACATAA TGAGGAAGCG TAATCAACAA AGGGCCCTAT GTTCTAGTGT CTGCCACTCC GGTGTCATT GGTGTCATT ACAAATAGCAG GCTGGGGCTC TGGTGGTTAC CTTTCTTCCC GCATCGCTTT AGGGTGTGAG TCGAGTCCAC TCTCGGCTCA ATGAGCTGAT GTGTGGAAAG AGTCAGCAAC TGCACTCTCA CTTCCGCCAG AGCCCGAGGC GCCTAGGCTT GCCTAGGCTT AGCAGGATG CCGCTTGGG TATTGGGCGA TATCCATCAT TCGACCAACA TCGATCAGGA GGCTCAAGGC TGCCGAATAT GTGTGGCGGA GCGGCGGAATG GCATCGGCTT

CATAATGCAT AAAACTCGAT CCACATATAT TGGTGCAATAA AATTTGTTT TCTATAGTGT CCAGCCATCT CATCTGCTCT TATCTGCGG GATGCTGGG TAGGGGTAT GCGCAGCGTG TTTCTTCTC AGGGTTCGGA TTACGTTAGT GTTCTTTTAT TTAACAATAA TCCCAGGCT CAGGTGTGGA TTAGTCAGCA TTTCCGCCAT CGCTCTGCC TTGCAAAAG AGGATCGTTT GGAGAGGCTA GTTCCGGCTG CTTGAATGAA TTGCGCAGCT AGTGCCGGG GGTGATGCA AGCGAAACAT TGATCTGGAC GCGCATGGCC CATGGTGGAA CCGCTATCAG GGTGACCCG CTTATCGGCTT

CAGGCTGGTA GTACTTCCGA AACCATTTAT TGCCACGCGAG TTAACATTTT CACCTAAATG GTTGTGTGCC TCCTAATAAA GGTGGGTGG GATGCGGTGG CCCACGCGC ACCGCTACAC GCCACGTTGG TTTAGTGCTT GGGCCATCGC AGTGACTCT TTAAGGGA TTAACGCGA CCCACGCGAG AAGTCCCGAG ACCATAGTCC TCTCCGCCCT TCTGAGCTAT CTCCCGGGAG CGCATGATTG TTCGGCTATG TCAGCGCAGG CTGCAAGGAG GTGCTCGAG CAGGATCTCC ATGCGCGGC CGCATCGAGC GAAGAGCAT GACGGCGAGG AATGGCCGT GACATAGCGT TTCTCTGTG CTTGACGAGT

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12960  
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13140  
13200  
13260  
13320  
13380  
13440  
13500  
13560  
13620  
13680

**FIG. 3G**

1374C	ACCTGCCCATC	GCAGCGCCCA	GACCGACCAA	GGTTCGAAAT	GGGACTCTGG	TCTTCTGAGC
1380C	TCGTTTTCCG	GGCTTCGGAA	TGAAAGGTTG	CCGCCCTTCTA	GATTCACCCG	GAATCCAGGATTC
1386C	TCGCCCAACCC	CTGGAGTTCT	GGATCTCATG	TCCAGCGCGG	TGGATGATCC	GGGAGGACGCCGGC
1392C	CAAAATTTTCA	AATAGCATCA	CAAAATAAGC	ATAATGGTTA	ATTGCAGCTT	ATTGCAGCTT
1398C	TCAATGTATC	TCCAAACTCA	TTGTGGTTTG	TGCATCTTAG	TTTTTTTCCAC	TTTTTTTCCAC
1404C	GGTCAAGCTT	GGCTAATCAT	CTAGAGCTTG	CGACCTCTAG	TGTAATCGTT	TTTATCATGTG
1410C	CGGGAAGCAT	AACATAACGAG	AATGCCACAC	ATCCGCTCAC	TGAAATGTT	TTTCTCTGTG
1416C	CGGTGCGCTC	ACATTAAATG	GAGCTAACTC	CTCAATGAGT	GCTTGGGGTG	GCCTGGGGTG
1422C	TCGGCCAACG	CATTAAATGAA	GTGCCAGCTG	GAAACCTGTC	TTCCAGTCGG	TTCCAGTCGG
1428C	CTGACTCGCT	TCCTCGCTCA	CTCTTCGGCT	GTAATGGGCG	GGCGTTTIGC	GGCGTTTIGC
1434C	TAAATACGGT	TCAAAGGGGG	ATCAGCTCAC	GGCAGCGGT	GTTCCGGCTGC	GTTCCGGCTGC
1440C	AGCAAAAGGC	GCAAAAGGCC	GAACATGTGA	ACGCAGGAAA	TCAGGGGATA	TCAGGGGATA
1446C	CCCCTGACGA	AGGCTCCGCC	GTITTTCCAT	CGTTGCTGGC	AAAAAGGCCG	AAAAAGGCCG
1452C	TATAAAGATA	CCGACAGGAC	GTGGCGAAAC	CAAGTCAGAG	AATCGACGCT	AATCGACGCT
1458C	TGCCGCTTAC	GTTCCGACCC	GCCTCTCCT	GCTCCCTCGT	CCCCCTGGAA	CCCCCTGGAA
1464C	GCTCAACGGTG	CTTCTCTCAAT	AGCGTGGCG	TCCCCTCGGG	TCCGCCTTTC	TCCGCCTTTC
1470C	ACGAAACCCCC	GGCTGTTGTC	CTCCAAGCTG	AGGTCGTTG	AGTTCGGTGT	AGTTCGGTGT
1476C	ACCCGGTAA	CTTGAGTCCA	TAACATATCT	CCTTATCCGG	GACCGCTGCG	GACCGCTGCG
1482C	CGAGGTATGT	ATTAGCAGAG	TGGTAACAGG	CAGCAGCCAC	TCGCCACTGG	TCGCCACTGG
1488C	GAAGGACAGT	GGCTACACTA	GCCTAACTAC	TGAAGTGGTG	ACAGAGTTCT	ACAGAGTTCT
1494C	GTAGCTCTTG	AAAAGAGTTG	TACCTTCGGA	TGAAGCCAGT	TGCCTCTGCG	TGCCTCTGCG
1500C	AGCAGATTAC	GTTCGAAGC	TGGTTTTTTT	CTGGTAGCGG	CAAAACCACCG	CAAAACCACCG
1506C	CTGACGCTCA	TCTACGGGGT	TTTGATCTTT	AAGAAGATCC	AAAGGATCTT	AAAGGATCTT
1512C	GGATCTTAC	TTATCAAAA	GGTCATGAGA	AAGGGATTTT	ACTCACETT	ACTCACETT
1518C	ATGAGTAAAC	TAAAGTATAT	TAAATCAATC	AATGAAGTTT	TTAAATTA	TTAAATTA
1524C	TCGTGCTATT	ATCTCAGGTA	TAGGCACTT	GCTTAATCAG	AGTTACCAAT	AGTTACCAAT
1530C	GGGAGGGCTT	ACTACGATAC	CGTGTAGATA	GACTCCCGT	ATAGTTGCTT	ATAGTTGCTT
1536C	CTCCAGATT	CGCTCACCGG	CGGAGACCCA	CAATGATACC	CCCAGTGCTG	CCCAGTGCTG
1542C	CAACTTTATC	AGTGGTCTTG	CGAGCGCAGA	CCGGAAGGGC	AACCAGCCAG	AACCAGCCAG
1548C	CGCCAGTTAA	GTAAGTAGTT	GGAAGCTAGA	ATTGTTGCCG	CAGTCTATTA	CAGTCTATTA
1554C	CGTCGTTTGG	GTTGACGCT	AGGCACTGTTG	CCAATGCTAC	AACGTTGTTG	AACGTTGTTG
1560C	CCCCCATGTT	GTTACATGAT	ATCAAGGCGA	GTTCCCAACG	TTCAGCTCCG	TTCAGCTCCG
1566C	AGTTGGCCCG	GTCAGAACTA	TCCGATCGTT	CCTTCCGGTCC	GGGTTAGCT	GGGTTAGCT
1572C	TGCCATCCGT	CTTACTGTCA	GCATAAATCT	TGGCAGCACT	CTCATGGTTA	CTCATGGTTA
1578C	AGTGTATCGG	TTCTGAGAA	GAAACAGTCA	GTGAGTACTC	TCTGTGACTG	TCTGTGACTG
1584C	ATAGCAGAAC	ACCGCGCCAC	ACGGGATAAT	CGGCGTCAAT	TGCTCTTGCC	TGCTCTTGCC
1590C	GGATCTTACC	AAACTCTCAA	TTCCGGGCCGA	GAAAACGTTT	CTCATCATTT	CTCATCATTT
1596C	CAGCATCTTT	AAC TGATCTT	TCGTGCAACC	TGTAACCCAC	TCCAGTTCGA	TCCAGTTCGA

FIG. 3H

TACTTTCACC AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAATGCGG CTTTTCCTT  
AATAAGGCG ACACGGAAAT GTTGAATACT CATACTCTTC GAATGTAAT CCTGACGTCG GCTTAAGTAA AGATCAAGT AGCATATCTG AGGATATCTG GACCTGAAAT TCGCGCGCTT  
CATTTATCAG GGTATTGTC TCATGAGCGG ATACATATTT GAATGTAAT CCTGACGTCG GCTTAAGTAA AGATCAAGT AGCATATCTG AGGATATCTG GACCTGAAAT TCGCGCGCTT  
ACAAATAGGG GTTCCGCGCA CATTTCCTCG TGGCAAGCTA AGAGAAGTTC ATCTGTGGTA TGGGCCAAAC TGGTCCCCCAG GTGCCCCCAAG TCGCTTCTGT TCGGGG  
AGATCTAATG AAAGACCCCA CCTGTAGGT AACTGAGAAT AGAGAAGTTC ATCTGTGGTA TGGGCCAAAC TGGTCCCCCAG GTGCCCCCAAG TCGCTTCTGT TCGGGG  
CAAGGCATGG AAAAATACAT GAATATGGG CAAACAGGAT TGGGCCAAAC TGGTCCCCCAG GTGCCCCCAAG TCGCTTCTGT TCGGGG  
TGGAACAGCT ACAGATGGAA CAGCTGAATA CAAGAACAGA TGTTTCCAGG AGTTTCGCTTC AACCCCTCAC  
AGGGCCAAGA GGCTCAGGGC CAAGAACAGA TGTTTCCAGG AGTTTCGCTTC AACCCCTCAC  
TTCCTGCCCC AACCAATCAGA AGTTTCGCTTC AACCCCTCAC  
GTTTCTAGAG CTAACCAATC AGTTTCGCTTC AACCCCTCAC  
CTTATTTGAA AAGAGCCAC AAGAGCCAC  
AGCTCAATAA AAGAGCCAC

16020 CAAATAAGGG  
16080 ATTTATTGAAG  
16140 AGAAATAA  
16200 ACGGATCGGG  
16260 CGCCATTTTG  
16320 CAGGAACAGA  
16380 GCCCGGCTC  
16440 TGGTAAGCAG  
16500 GCCCTCAGCA  
16560 GACCCCTGTGC  
16620 CTGCTCCCCG  
16656

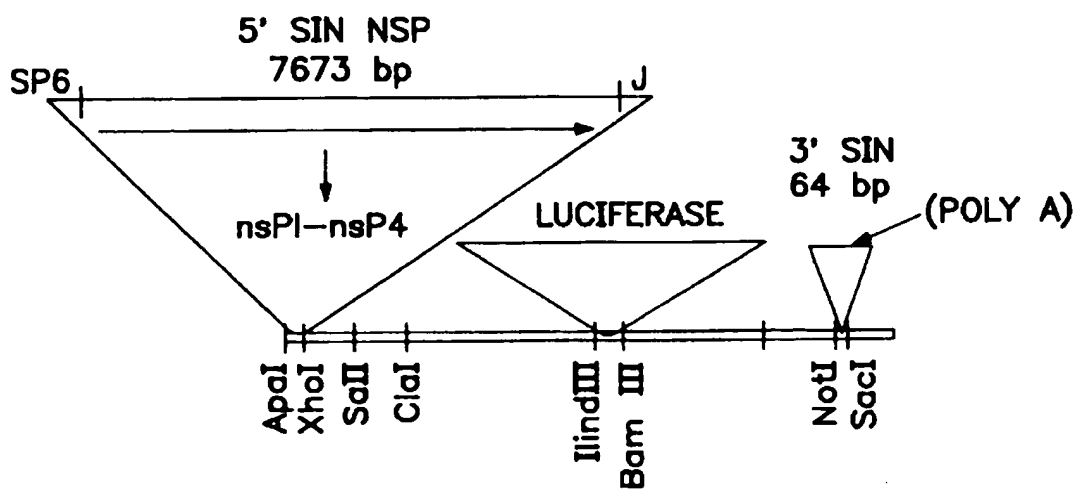
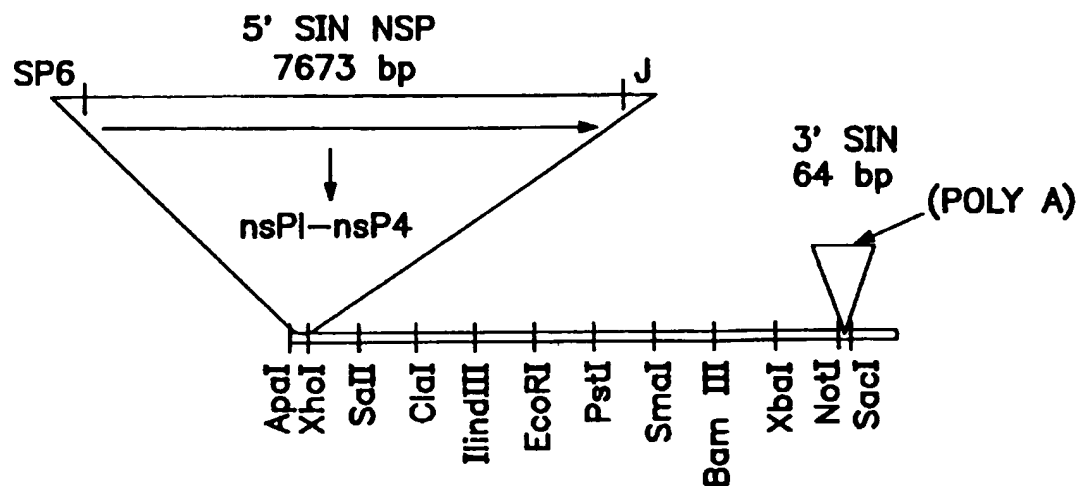


FIG. 4



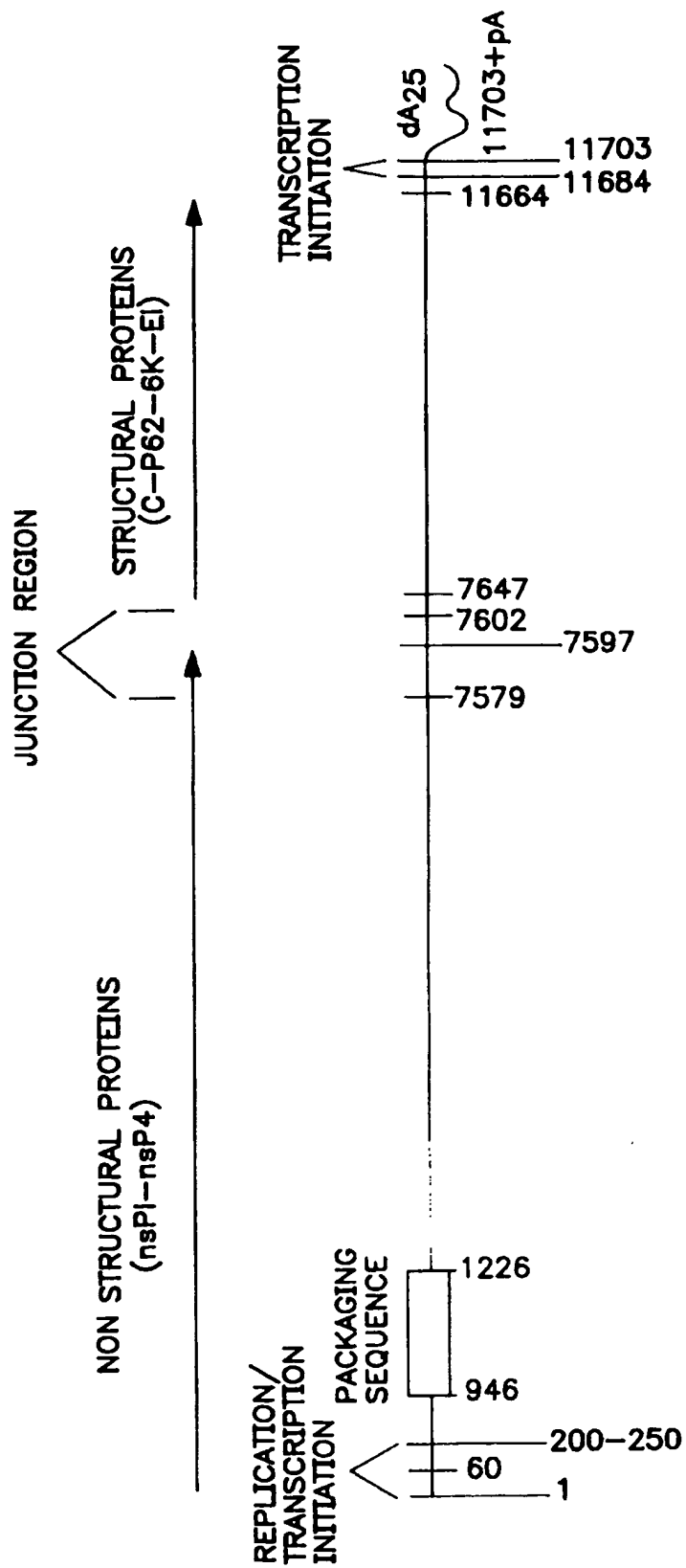


FIG. 5A

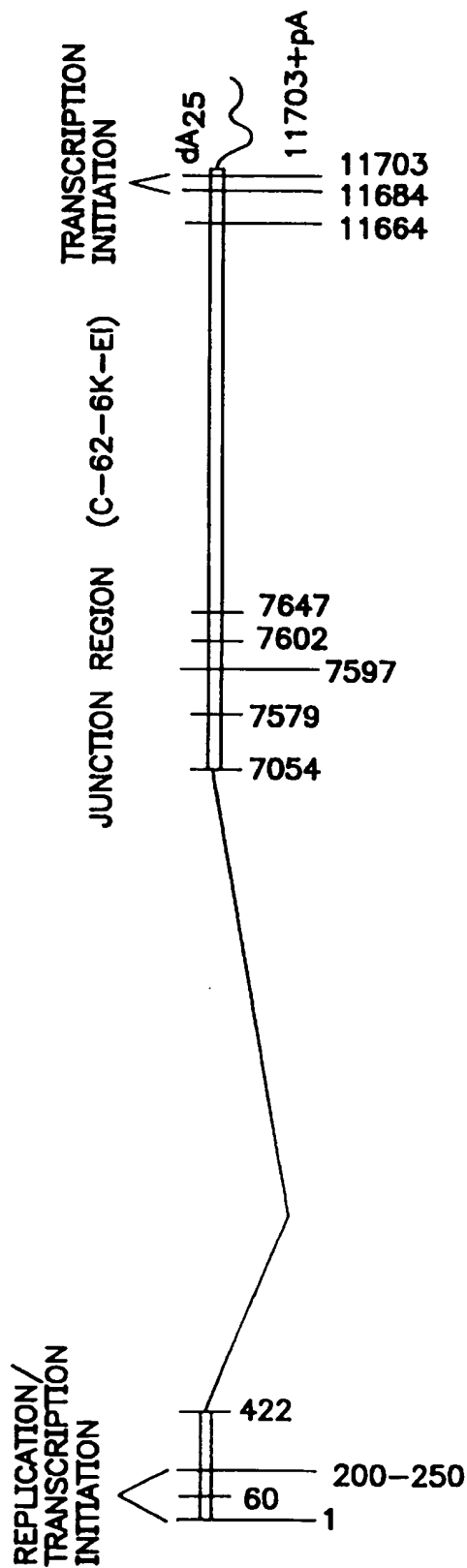


FIG. 5B

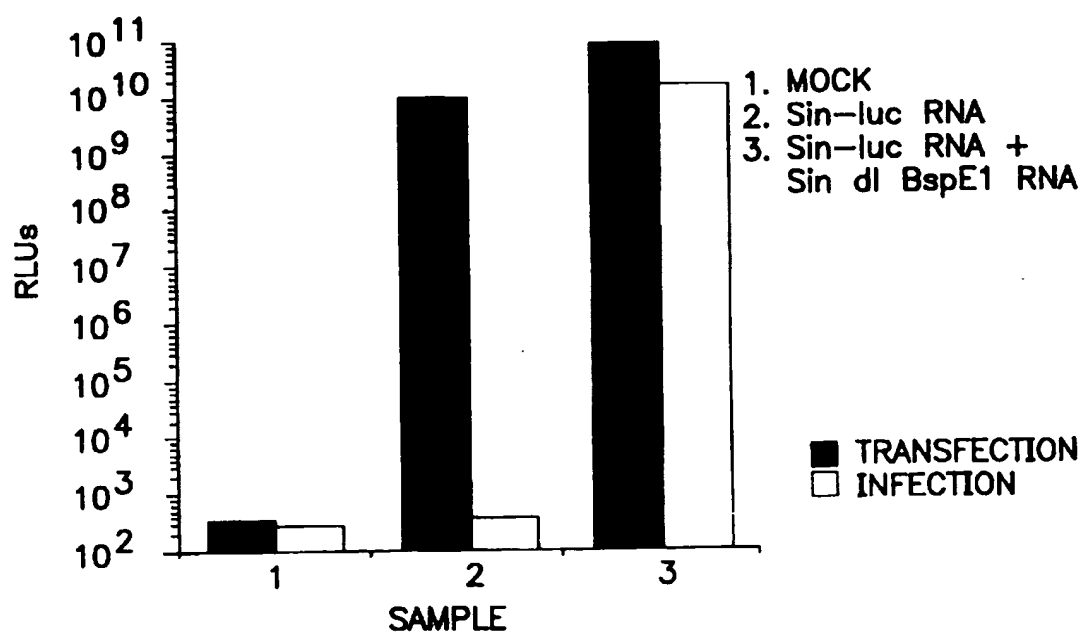
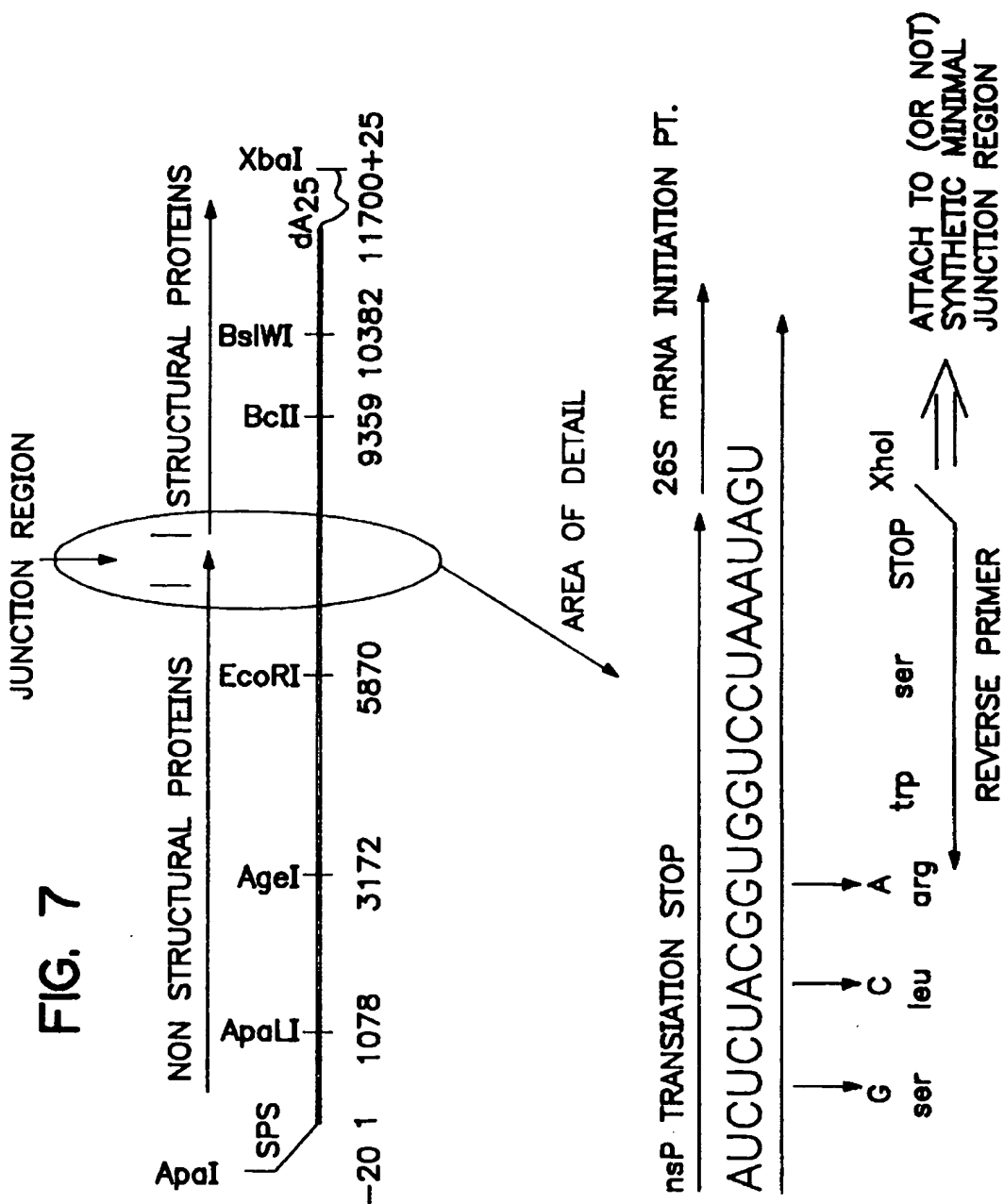


FIG. 6



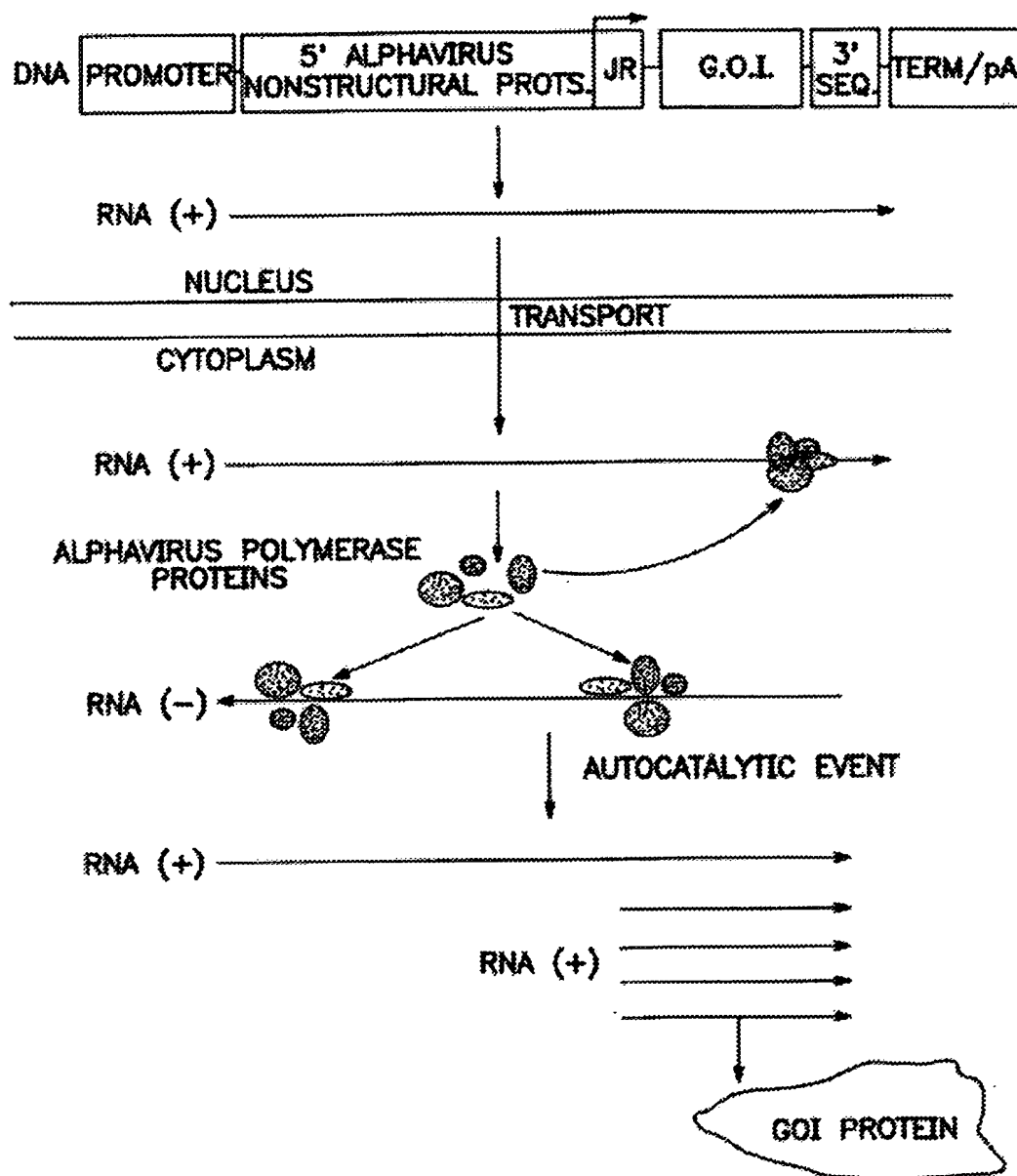


FIG. 8

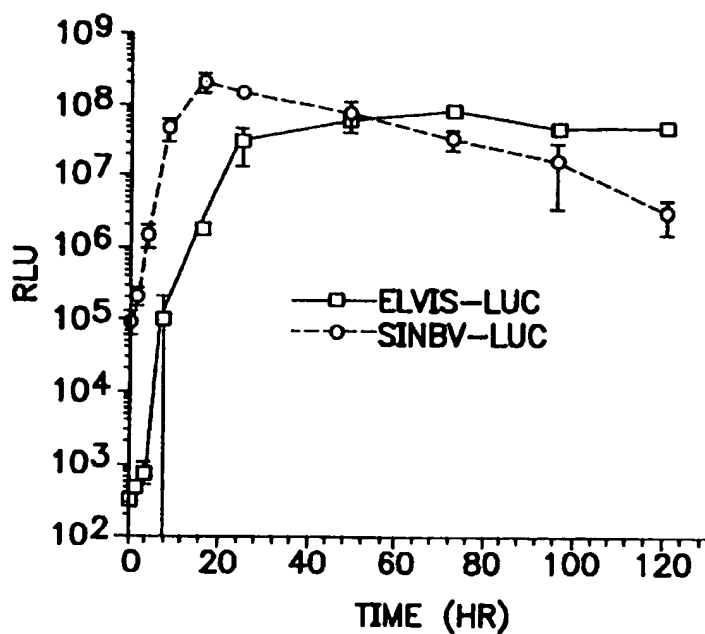


FIG. 9

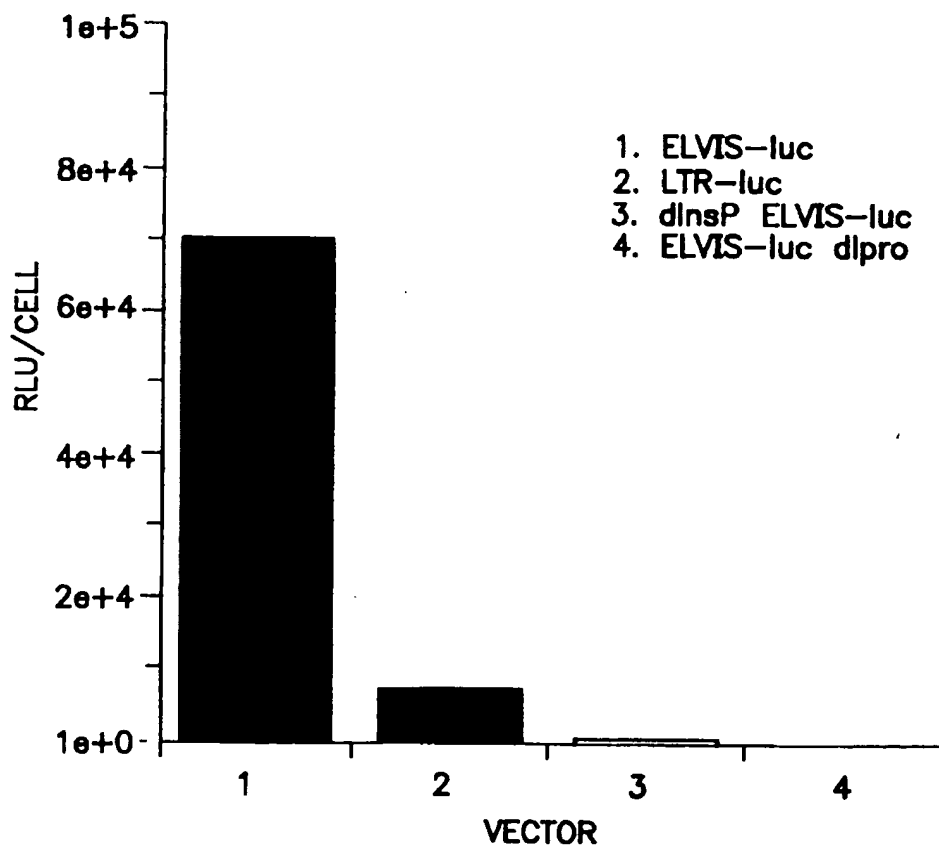


FIG. 10

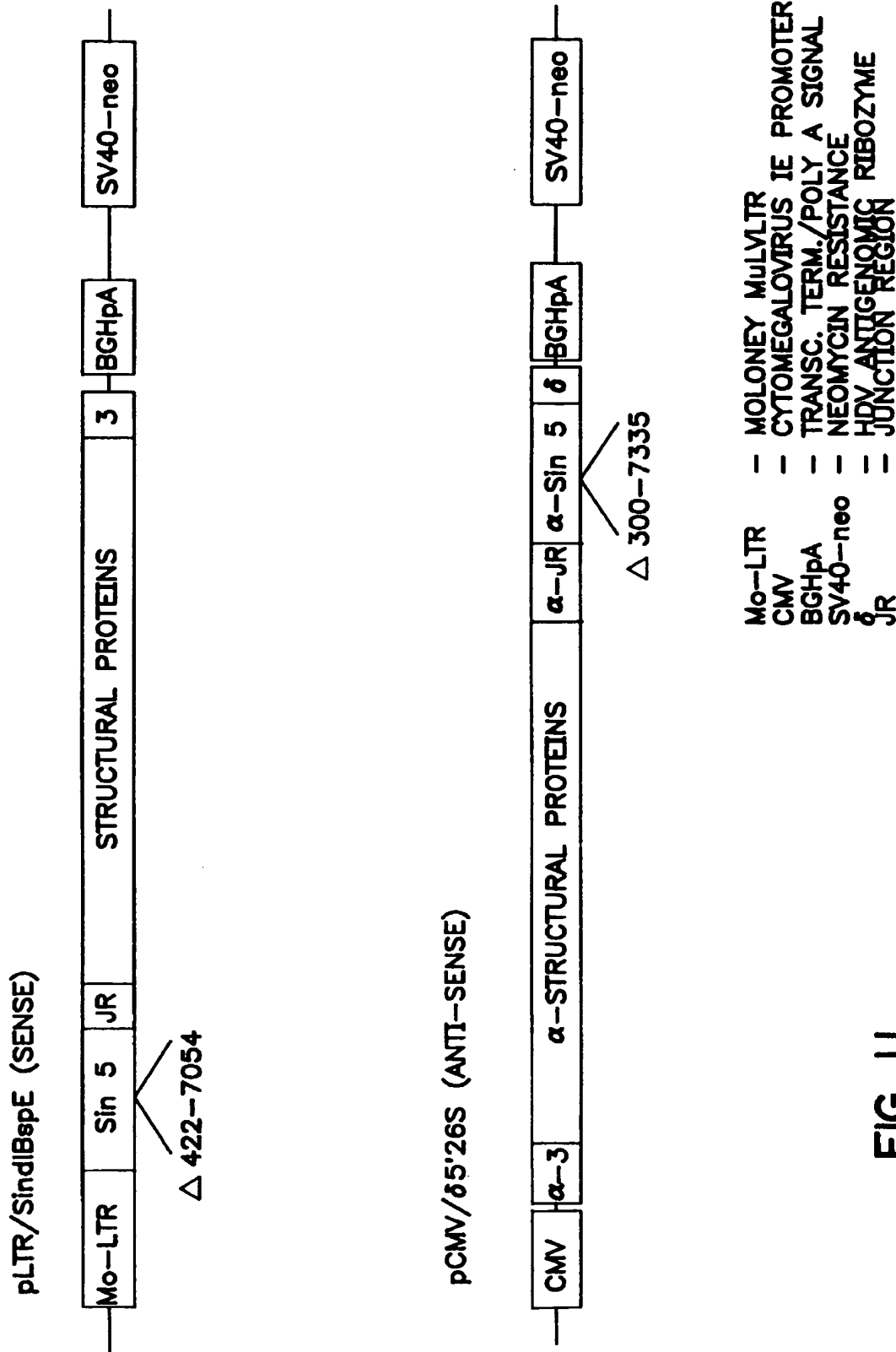


FIG. 11

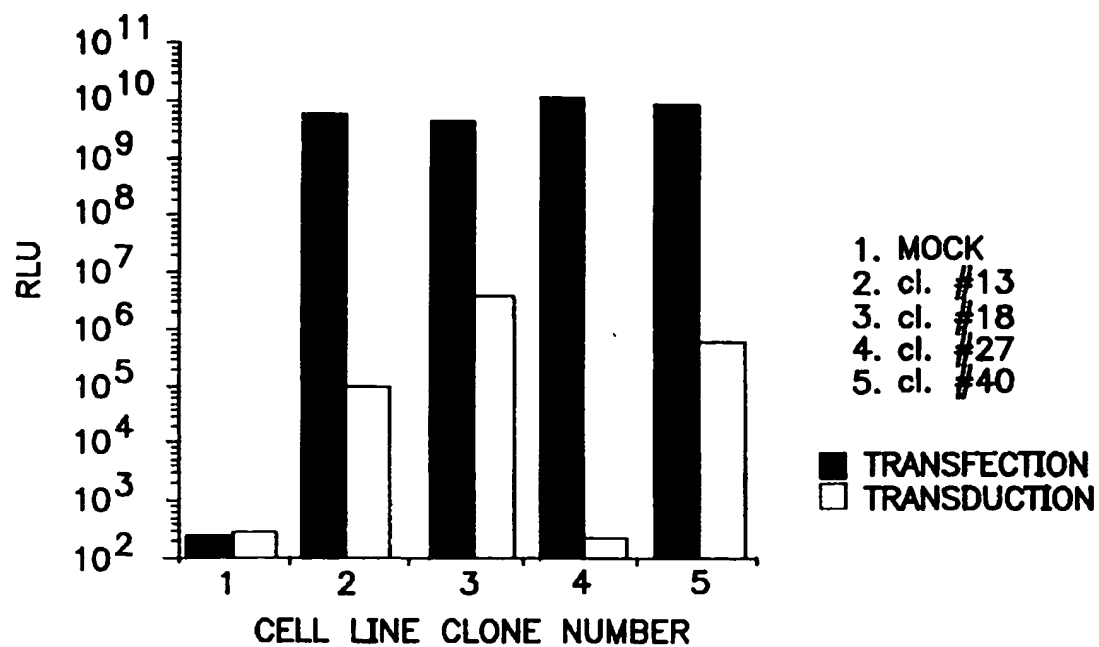


FIG. 12

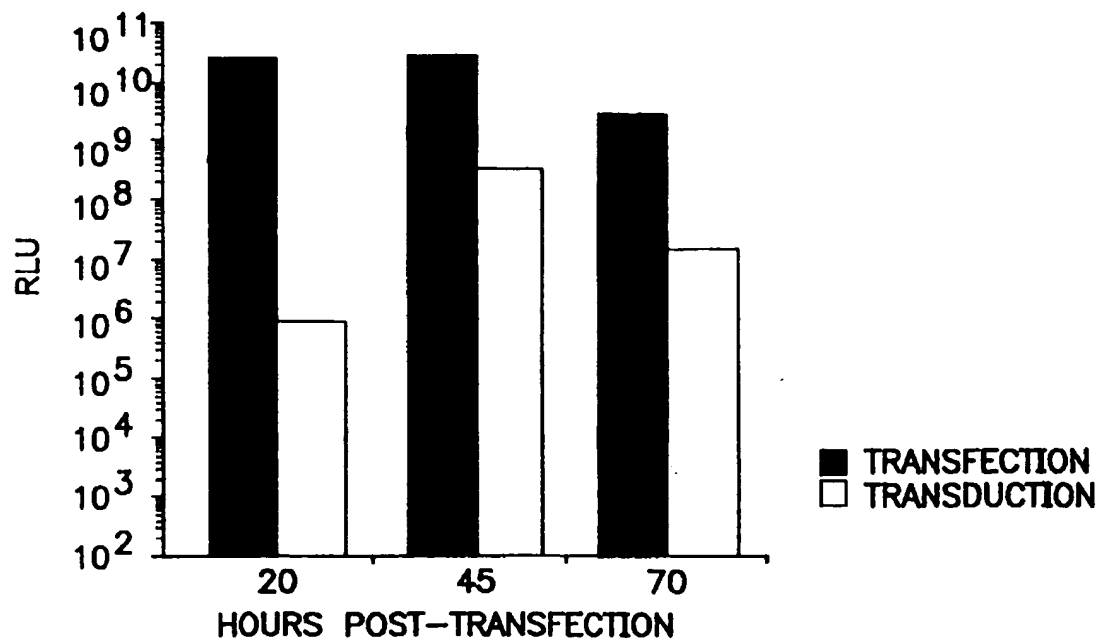


FIG. 13



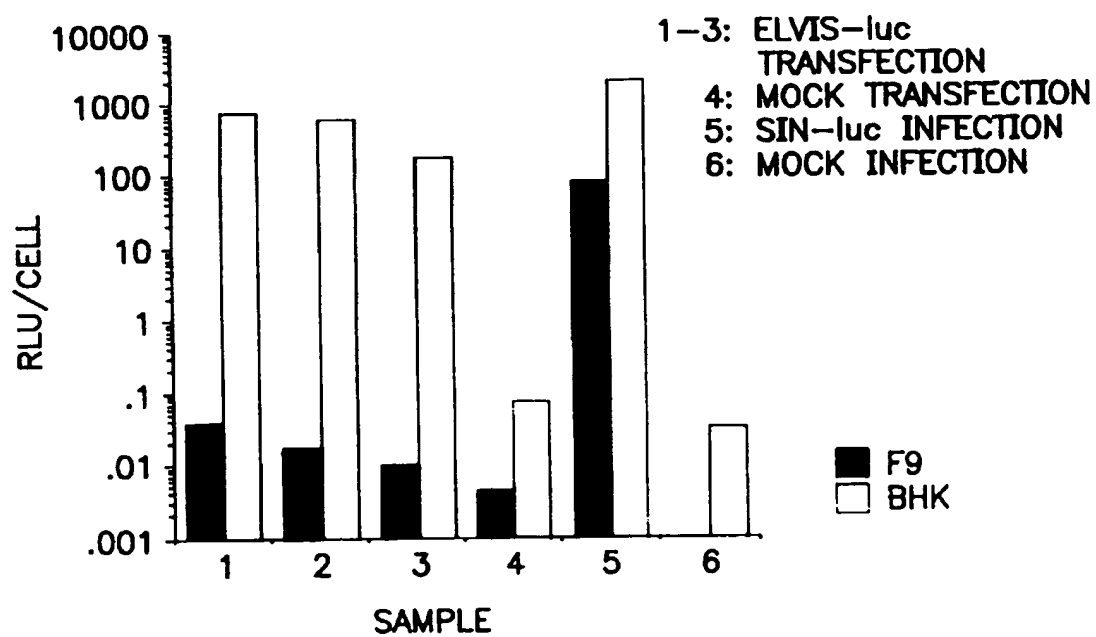


FIG. 14

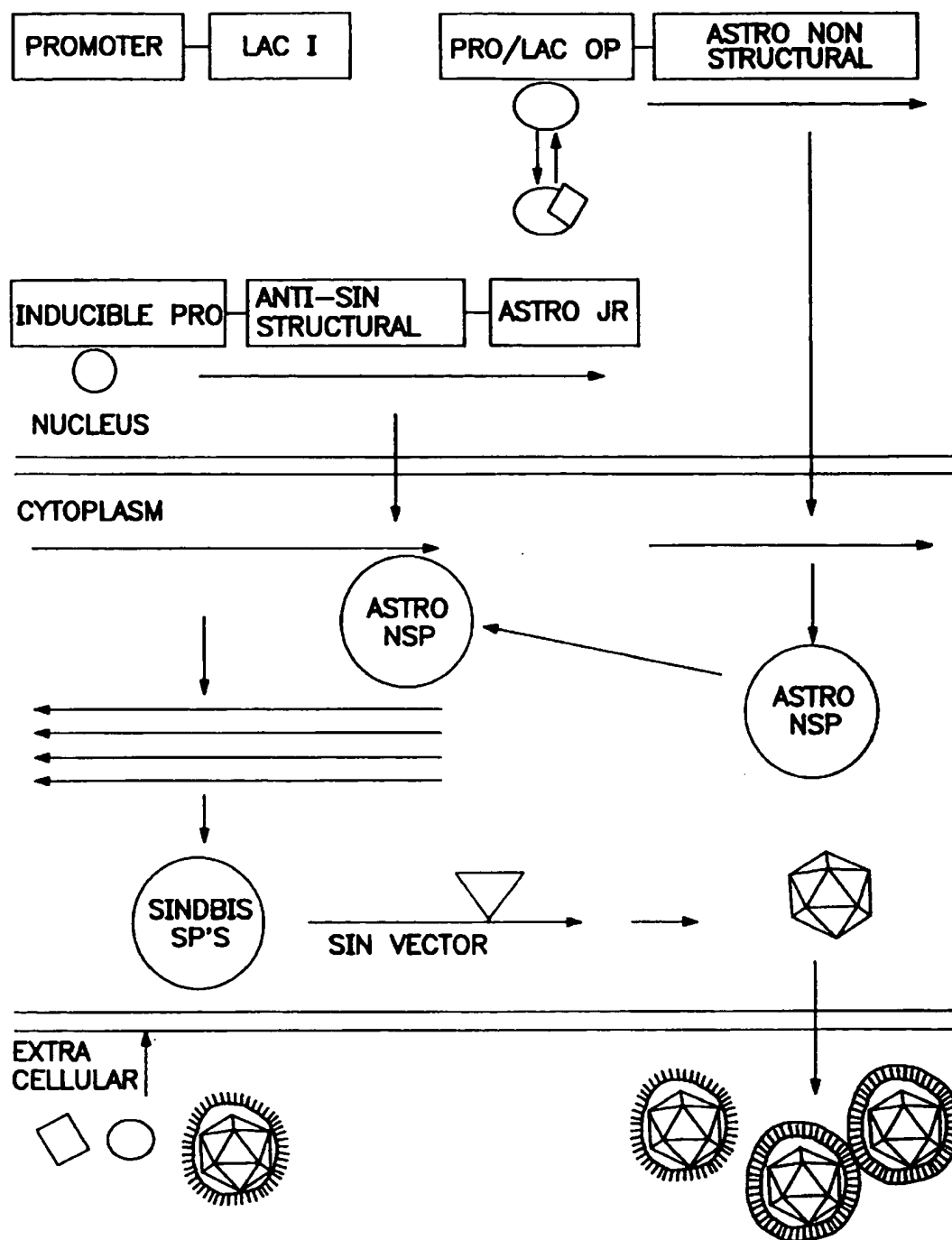


FIG. 15

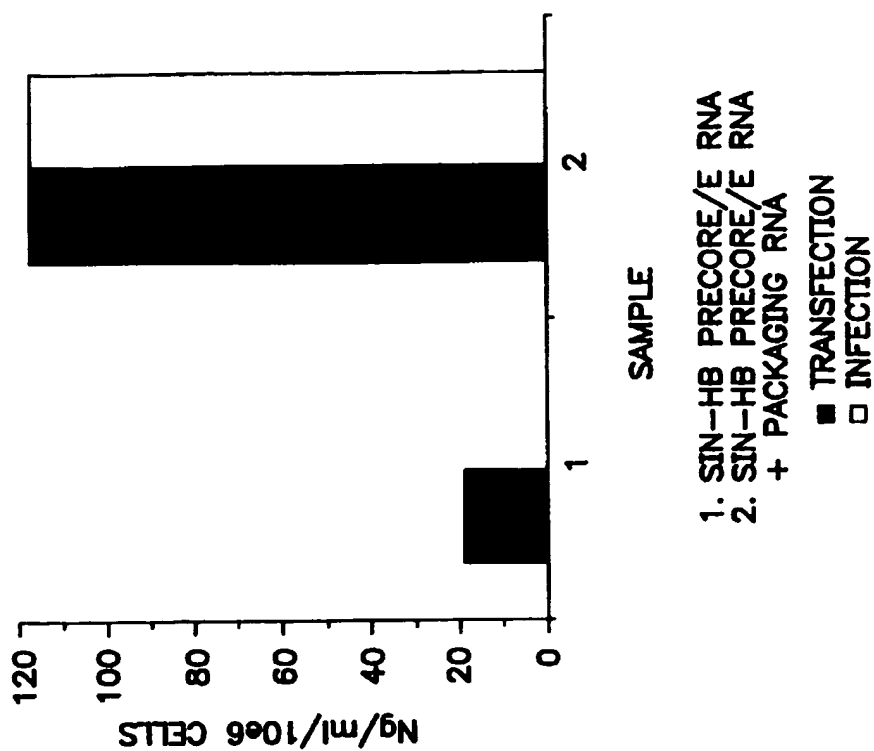


FIG. 16B

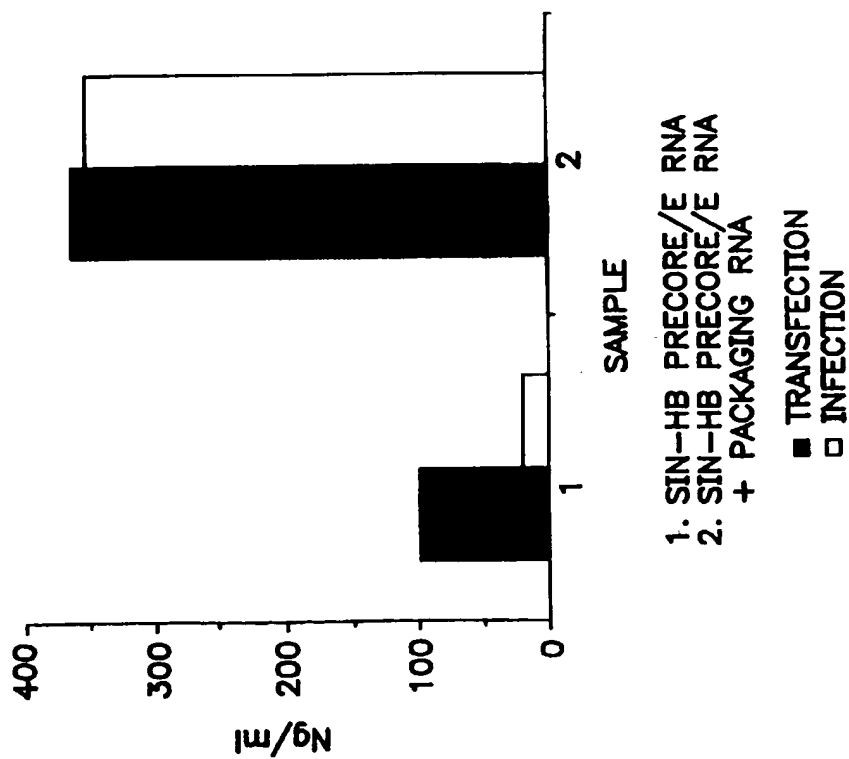


FIG. 16A

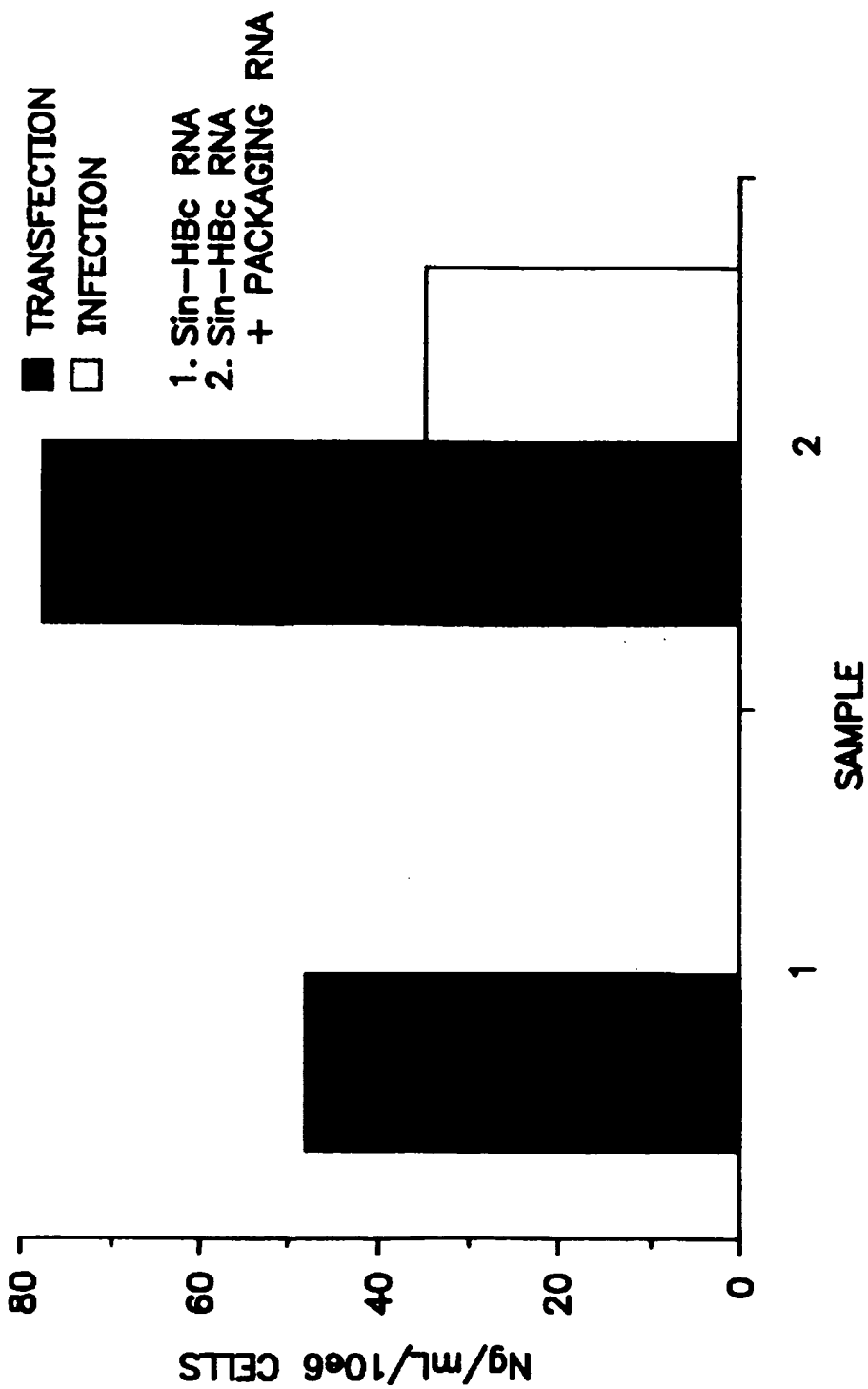


FIG. 17

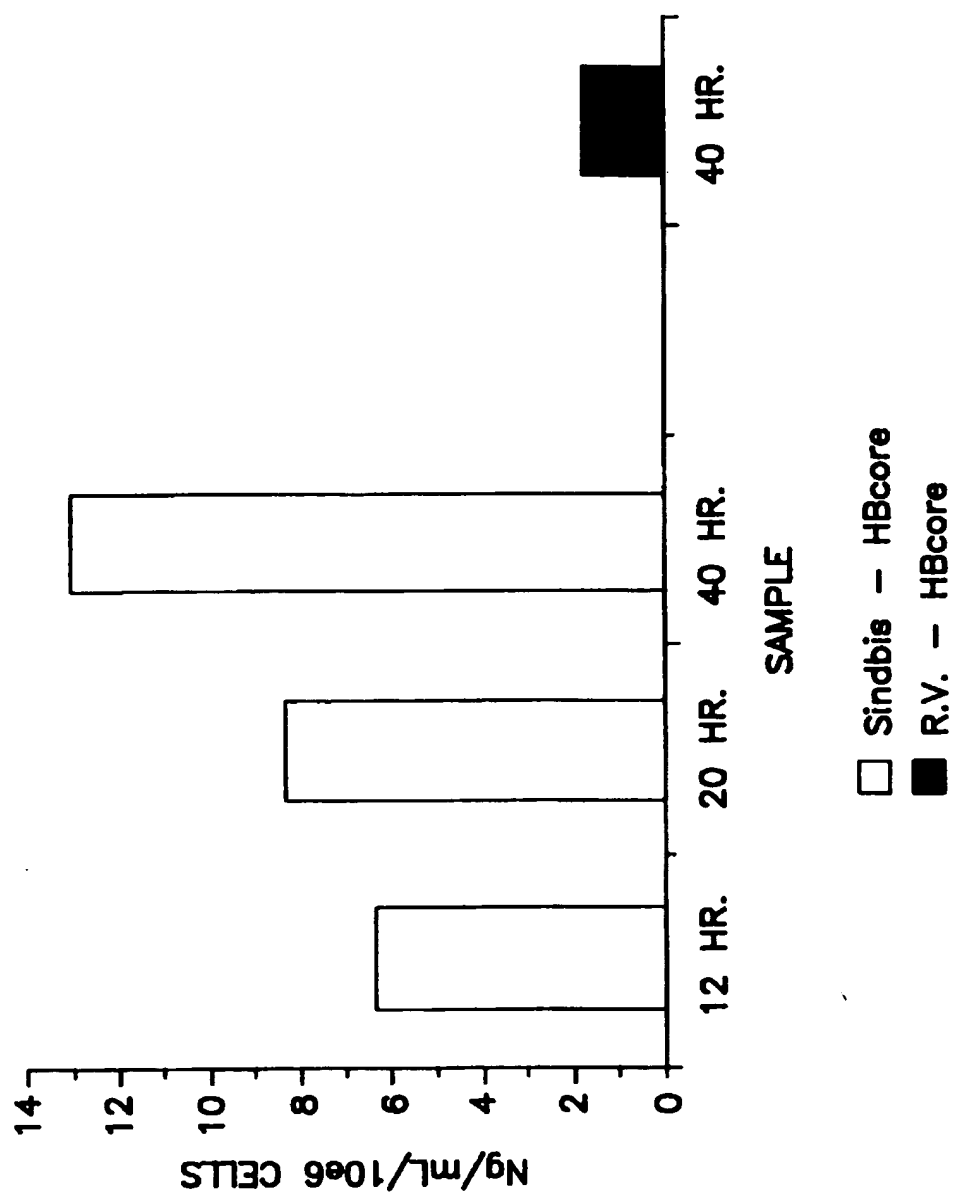


FIG. 18

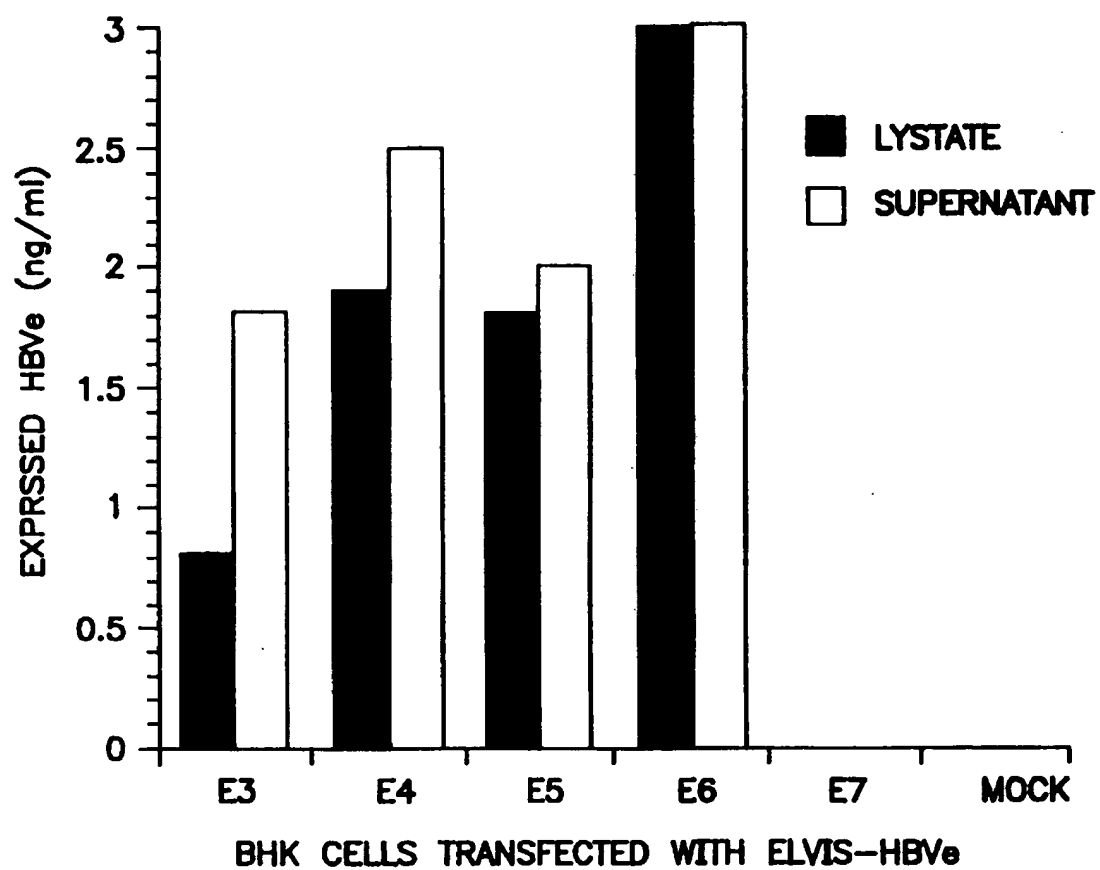
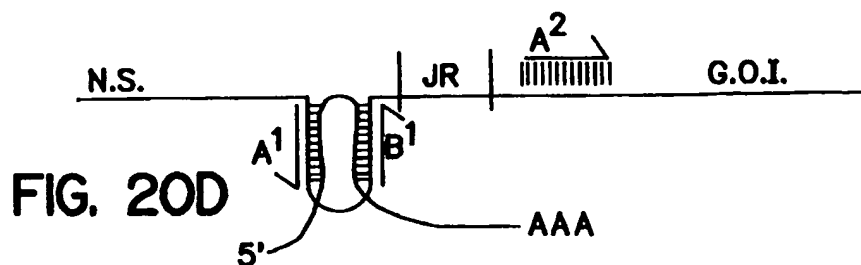
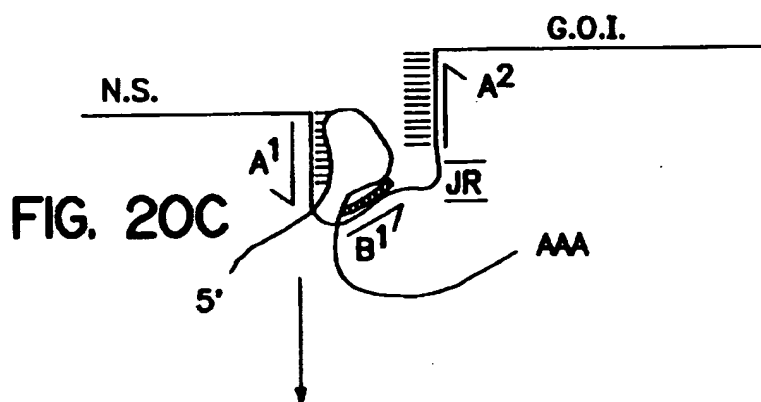
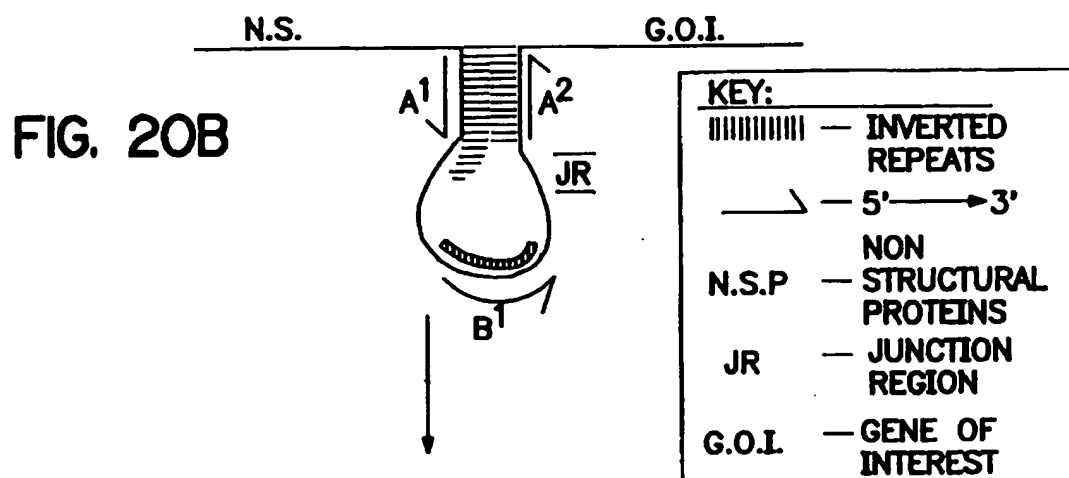
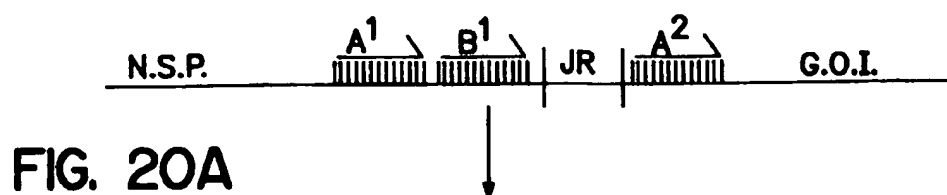


FIG. 19



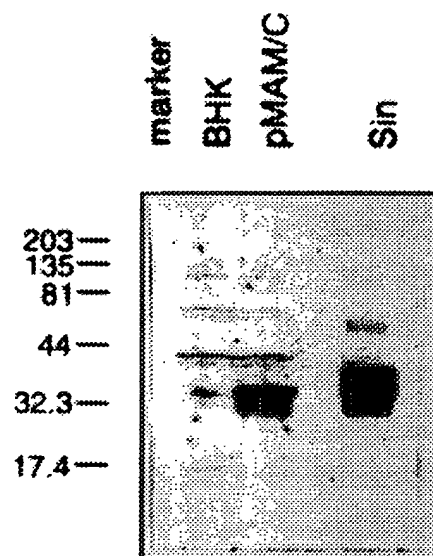


FIG. 21



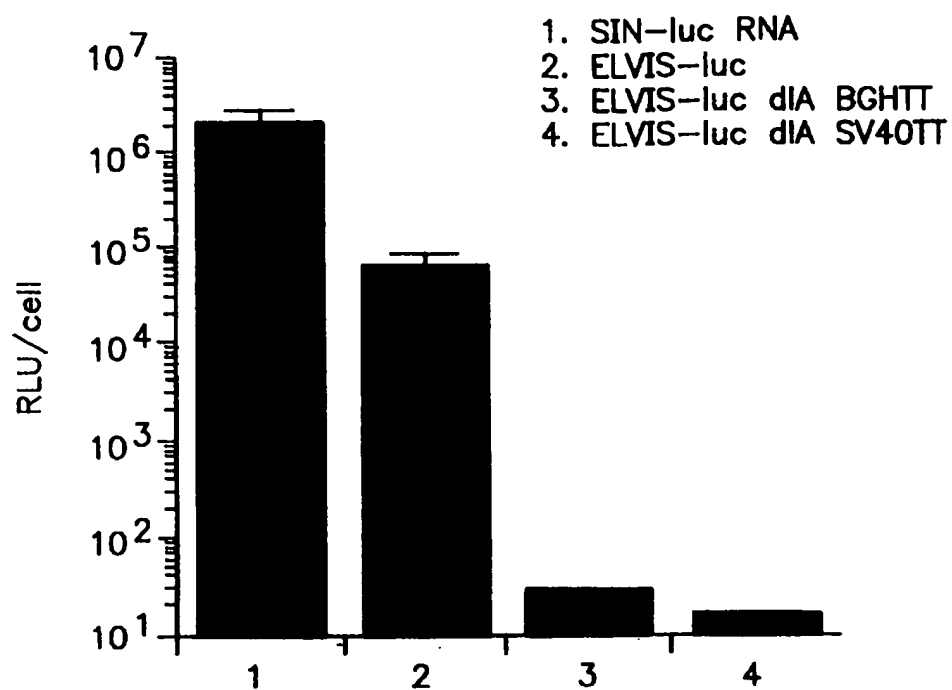


FIG. 22A

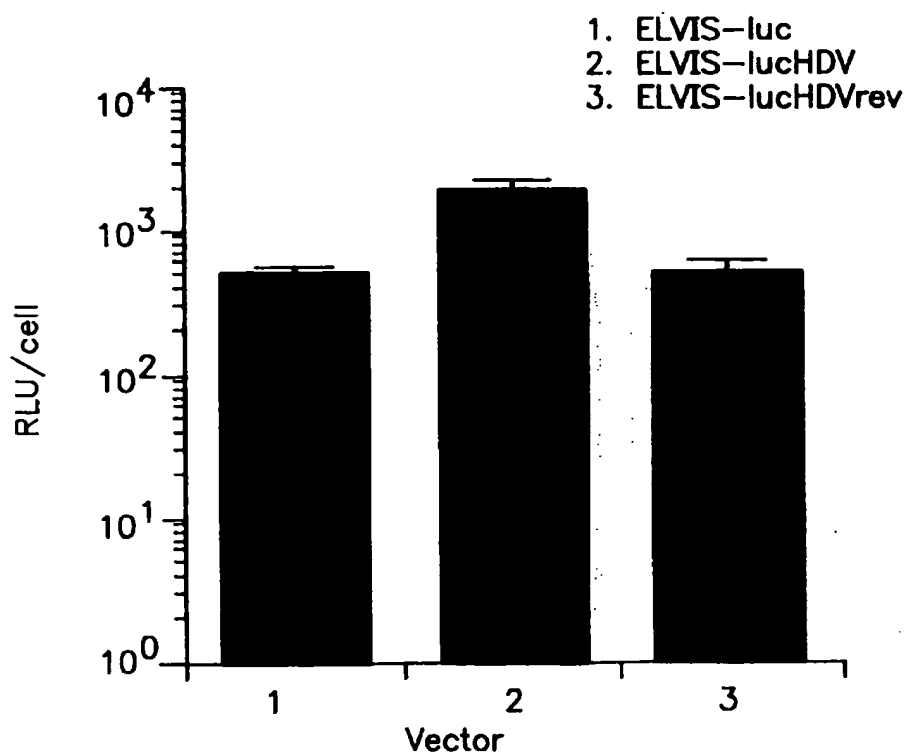


FIG. 22B

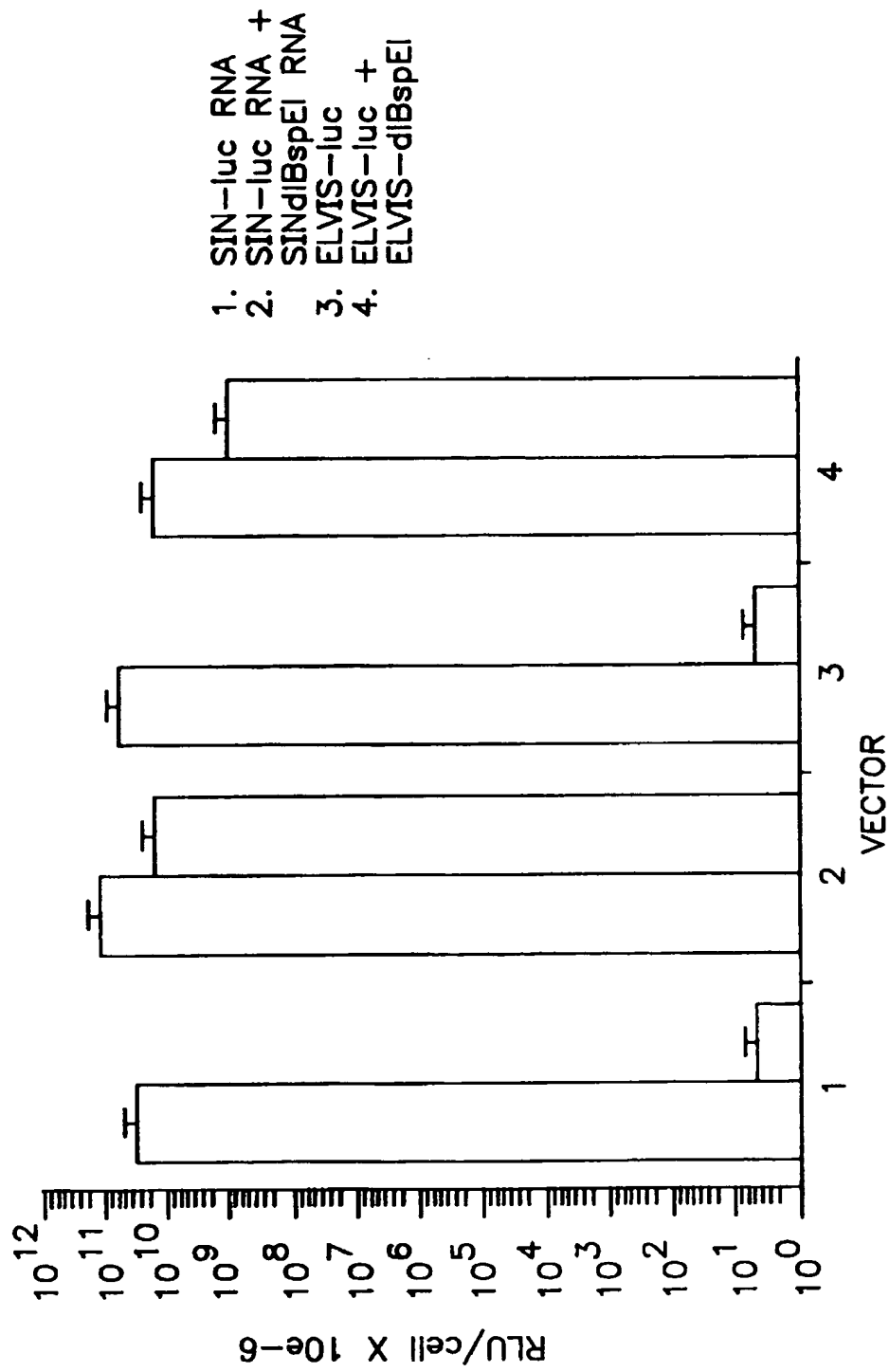
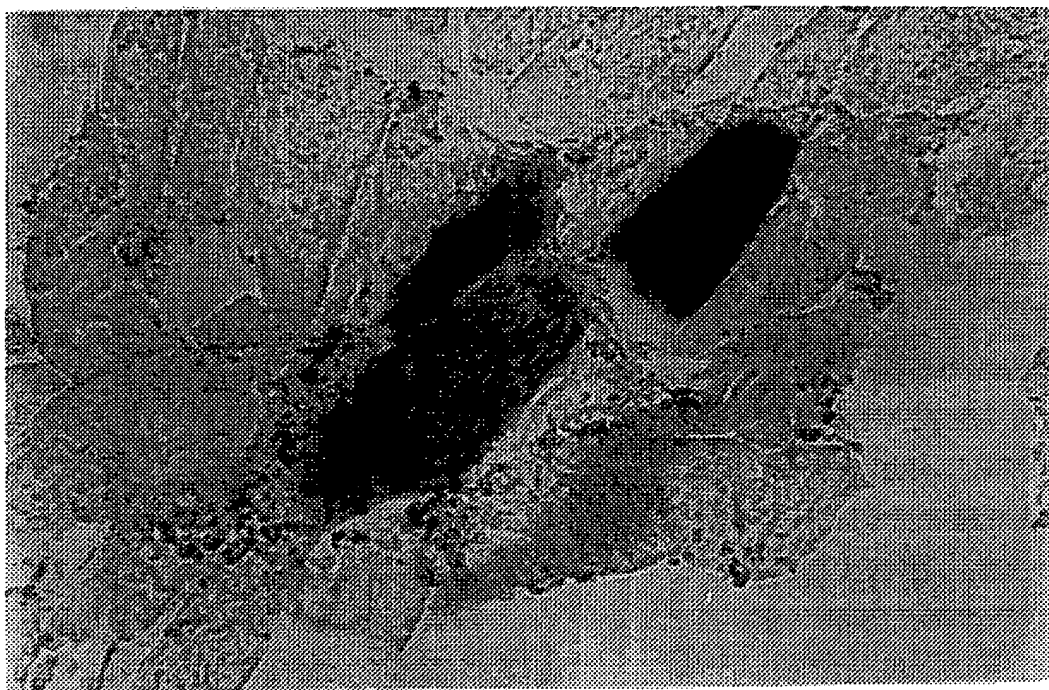


FIG. 23



**FIG. 24**

## ALPHAVIRUS VECTOR CONSTRUCTS

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of copending U.S. patent application Ser. No. 08/404,796, filed Mar. 20, 1995; which application is a continuation-in-part of U.S. patent application Ser. No. 08/376,184, filed Jan. 20, 1995, now abandoned; which application is a continuation-in-part of U.S. patent application Ser. No. 08/348,472, filed Nov. 30, 1994, now abandoned; which application is a continuation in part of U.S. patent application Ser. No. 08/198,450, filed Feb. 18, 1994, now abandoned; which application is a continuation-in-part of U.S. patent application Ser. No. 08/122,791, filed Sep. 15, 1993, now abandoned.

## TECHNICAL FIELD

The present invention relates generally to use of recombinant viruses as vectors, and more specifically, to recombinant alphaviruses which are capable of expressing a heterologous sequence in target cells.

## BACKGROUND OF THE INVENTION

Alphaviruses comprise a set of serologically related arthropod-borne viruses of the Togavirus family. Briefly, alphaviruses are distributed worldwide, and persist in nature through a mosquito to vertebrate cycle. Birds, rodents, horses, primates, and humans are among the defined alphavirus vertebrate reservoir/hosts.

Twenty-six known viruses and virus subtypes have been classified within the alphavirus genus utilizing the hemagglutination inhibition (HI) assay. Briefly, the HI test segregates the 26 alphaviruses into three major complexes: the Venezuelan encephalitis (VE) complex, the Semliki Forest (SF) complex, and the western encephalitis (WE) complex. In addition, four additional viruses, eastern encephalitis (EE), Barmah Forest, Middelburg, and Ndumu, receive individual classification based on the HI serological assay.

Members of the alphavirus genus are also classified based on their relative clinical features in humans: alphaviruses associated primarily with encephalitis, and alphaviruses associated primarily with fever, rash, and polyarthritides. Included in the former group are the VE and WE complexes, and EE. In general, infection with this group can result in permanent sequelae, including behavior changes and learning disabilities, or death. In the latter group is the SF complex, comprised of the individual alphaviruses Chikungunya, O'nyong-nyong, Sindbis, Ross River, and Mayaro. With respect to this group, although serious epidemics have been reported, infection is in general self-limiting, without permanent sequelae.

Sindbis virus is the prototype member of the alphavirus genus of the Togavirus family. Although not usually apparent, clinical manifestations of Sindbis virus infection may include fever, arthritis, and rash. Sindbis virus is distributed over Europe, Africa, Asia, and Australia, with the best epidemiological data coming from South Africa, where 20% of the population is seropositive. (For a review, see Peters and Dalrymple, *Fields Virology* (2d ed), Fields et al. (eds.), B.N. Raven Press, New York, N.Y., chapter 26, pp. 713-762). Infectious Sindbis virus has been isolated from human serum only during an outbreak in Uganda and in a single case from Central Africa.

The morphology and morphogenesis of the alphavirus genus is generally quite uniform. In particular, the enveloped

60-65 nm particles infect most vertebrate cells, where productive infection is cytopathic. On the other hand, infection of invertebrate cells, for example, those derived from mosquitoes, does not result in any overt cytopathology.

Typically, alphaviruses are propagated in BHK-21 or vero cells, where growth is rapid, reaching a maximum yield within 24 hours of infection. Field strains are usually isolated on primary avian embryo, for example chicken fibroblast cultures (CEF).

The genomic RNA (49S RNA) of alphaviruses is unsegmented, of positive polarity, approximately 11-12 kb in length, and contains a 5' cap and a 3' polyadenylate tail. Infectious enveloped virus is produced by assembly of the viral nucleocapsid proteins onto genomic RNA in the cytoplasm, and budding through the cell membrane embedded with viral-encoded glycoproteins. Entry of virus into cells appears to occur by endocytosis through clathrin-coated pits, fusion of the viral membrane with the endosome, release of the nucleocapsid and uncoating of the viral genome. During viral replication, the genomic 49S RNA serves as template for synthesis of a complementary negative strand. The negative strand in turn serves as template for full-length genomic RNA and for an internally initiated positive-strand 26S subgenomic RNA. The non-structural proteins are translated from the genomic RNA. Alphaviral structural proteins are translated from the subgenomic 26S RNA. All viral genes are expressed as polypeptides and processed into individual proteins by proteolytic cleavage post-translation.

The use of recombinant virus vectors (in particular, alphavirus vectors) to treat individuals requires that they be able to be transported and stored for long periods at a desired temperature, such that infectivity and viability of the recombinant virus is retained. Current methods for storing recombinant viruses generally involve storage as liquids and at low temperatures. Such methods present problems in Third World countries, which typically do not have adequate refrigeration capabilities. For example, each year in Africa, millions of children die from infectious diseases such as measles. Vaccines necessary for the prevention of these diseases cannot be distributed to the majority of these countries because refrigeration is not readily accessible.

In addition to storage as liquids and at low temperatures, present viral formulations often contain media components that are not desirable for injection into patients. Consequently, there is a need in the art for a method of preserving purified recombinant viral vector (and in particular, alphavirus vectors) in a lyophilized form at elevated temperatures, and for this form to be suitable for injection into patients.

The present invention discloses recombinant alphavirus vectors which are suitable for use in a variety of applications, including for example, gene therapy, and further provides other related advantages.

## SUMMARY OF THE INVENTION

Briefly stated, the present invention provides alphavirus vector constructs and alphavirus particles, as well as methods of making and utilizing the same. Within one aspect of the present invention, alphavirus vector constructs are provided comprising a 5' promoter which is capable of initiating the synthesis of viral RNA in vitro from cDNA, a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-structural proteins, a viral junction region which has been inactivated such that viral transcription of the subgenomic

fragment is prevented, and an alphavirus RNA polymerase recognition sequence. Within other aspects of the present invention, the viral junction region has been modified such that viral transcription of the subgenomic fragment is reduced.

Within yet other aspects of the present invention, alphavirus vector constructs are provided comprising a 5' promoter which is capable of initiating the synthesis of viral RNA in vitro from cDNA, a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-structural proteins, a first viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, a second viral junction region which is active, or which has been modified such that viral transcription of the subgenomic fragment is reduced, and an alphavirus RNA polymerase recognition sequence.

Within still other aspects of the present invention, alphavirus cDNA vector constructs are provided, comprising a 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA, followed by a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-structural proteins, a viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription termination.

Within another aspect of the present invention, alphavirus cDNA vector constructs are provided, comprising a 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA, followed by a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-structural proteins, a viral junction region which is active, or which has been modified such that viral transcription of the subgenomic fragment is reduced, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription termination.

Within another aspect of the present invention, alphavirus cDNA vector constructs are provided, comprising a promoter which is capable of initiating the synthesis of viral RNA from cDNA followed by a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-structural proteins, a first viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, followed by a second viral junction region which is active, or which has been modified such that viral transcription of the subgenomic fragment is reduced, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription termination.

Within other aspects of the present invention, eukaryotic layered vector initiation systems are provided which are capable of expressing a heterologous nucleic acid sequence in a eukaryotic cell transformed or transfected therewith. In particular embodiments, eukaryotic layered vector initiation systems are provided, comprising a promoter which is capable of initiating the 5' synthesis of RNA from cDNA, a vector construct which is capable of autonomous replication in a cell, the vector construct being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription termination.

Within a related aspect, eukaryotic layered vector initiation systems are provided, comprising a DNA promoter which is capable of initiating the 5' synthesis of RNA from cDNA, a vector construct which is capable of autonomous

replication in a cell, the vector construct being capable of expressing a heterologous ribonucleic acid sequence, and a 3' DNA sequence which controls transcription termination.

Within one embodiment, the vector construct within the eukaryotic layered vector initiation systems of the present invention is an alphavirus vector construct. Within other embodiments, the construct is derived from a virus selected from the group consisting of poliovirus, rhinovirus, coxsackieviruses, rubella, yellow fever, HCV, TGEV, IBV, MHV, BCV, parainfluenza virus, mumps virus, measles virus, respiratory syncytial virus, influenza virus, RSV, MoMLV, HIV, HTLV, hepatitis delta virus and Astrovirus. Within yet other embodiments, the promoter which is capable of initiating the 5' synthesis of RNA from cDNA is selected from the group consisting of the MoMLV promoter, metallothionein promoter, glucocorticoid promoter, SV40 promoter, and the CMV promoter. Within further embodiments, the eukaryotic layered vector initiation systems further comprise a polyadenylation sequence.

In further embodiments of the invention, in any of the above aspects, the vectors (e.g., alphavirus vector construct, alphavirus cDNA vector construct, or eukaryotic layered vector initiation system) may be derived from an alphavirus selected from the group consisting of Aura, Fort Morgan, Venezuelan Equine Encephalitis, Ross River, Semliki Forest, Sindbis, and Mayaro.

In other embodiments, the vectors described above contain a heterologous sequence. Typically, such vectors contain a heterologous nucleotide sequence of greater than 100 bases, generally the heterologous nucleotide sequence is greater than 3 kb, and sometimes greater than 5 kb, or even 8 kb. In various embodiments, the heterologous sequence is a sequence encoding a protein selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, alpha-, beta-, or gamma-IFN, G-CSF, and GM-CSF. Within other embodiments of the invention, the heterologous sequence may encode a lymphokine receptor. Representative examples of such receptors include receptors for any of the lymphokines set forth above.

In still other embodiments, the vectors described above include a selected heterologous sequence which may be obtained from a virus selected from the group consisting of influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II and CMV. Within one preferred embodiment, the heterologous sequence obtained from HPV encodes a protein selected from the group consisting of E5, E6, E7 and L1. In yet other embodiments, the vectors described above include a selected heterologous sequence encoding an HIV protein selected from the group consisting of HIV gp120 and gag.

The selected heterologous sequences described above also may be an antisense sequence, noncoding sense sequence, or ribozyme sequence. In preferred embodiments, the antisense or noncoding sense sequence is selected from the group consisting of sequences which are complementary to influenza virus, HPV, HBV, HCV, EBV, HIV, HSV, FeLV, FIV, Hanta virus, HTLV I, HTLV II, and CMV sequences.

In another embodiment, the vectors described above contain no alphavirus structural protein genes. Within other embodiments, the selected heterologous sequence is located downstream from a viral junction region. In the vectors described above having a second viral junction, the selected heterologous sequence may, within certain embodiments, be located downstream from the second viral junction region. Where the heterologous sequence is located downstream

from a viral junction region, the vector construct may further comprise a polylinker located subsequent to the viral junction region. Within preferred embodiments, such polylinkers do not contain a restriction endonuclease recognition sequence present in the wild-type alphavirus sequence.

In yet another embodiment, in the vectors described above the selected heterologous sequence may be located within the nucleotide sequence encoding alphavirus non-structural proteins.

In particular embodiments, the vectors described above include a viral junction region consisting of the nucleotide sequence as shown in FIG. 3, from nucleotide number 7579, to nucleotide number 7597 (SEQ. ID NO. 1). In alternative embodiments, where the vector includes a second viral junction, an E3 adenovirus gene may be located downstream from the second viral junction region. Vectors of the present invention may also further comprise a non-alphavirus (for example retrovirus, coronavirus, hepatitis B virus) packaging sequence located between the first viral junction region and the second viral junction region, or in the nonstructural protein coding region.

In further aspects, the present invention provides an isolated recombinant alphavirus vector which does not contain a functional viral junction region, and which in preferred embodiments produces reduced viral transcription of the subgenomic fragment.

In still a further aspect, the present invention provides an alphavirus structural protein expression cassette, comprising a promoter and one or more alphavirus structural protein genes, the promoter being capable of directing the expression of alphavirus structural proteins. In various embodiments, the expression cassette is capable of expressing alphavirus structural proteins, such as an alphavirus structural protein selected from the group consisting of C, 6K, E3, E2, and E1.

Within other embodiments, the alphavirus structural protein is derived from an alphavirus selected from the group consisting of Aura, Fort Morgan, Venezuelan Equine Encephalitis, Ross River, Semliki Forest, Sindbis and Mayaro viruses.

In yet another aspect, the present invention provides an alphavirus structural protein expression cassette, comprising a promoter, one or more alphavirus structural proteins, and a heterologous ligand sequence, the promoter being capable of directing the expression of the alphavirus structural proteins and the heterologous sequence. In various embodiments, the heterologous ligand sequence is selected from the group consisting of VSVG, HIV gp120, antibody, insulin, and CD4.

In certain embodiments, the expression cassettes described above include a promoter selected from the group consisting of metallothionein, Drosophila actin 5C distal, SV40, heat shock protein 65, heat shock protein 70, Py, RSV, BK, JC, MuLV, MMTV, alphavirus junction region, CMV and VA1RNA.

The present invention also provides packaging cell lines and producer cell lines suitable for producing recombinant alphavirus particles. Such packaging or producer cell lines may be either mammalian or non-mammalian (e.g., insect cells, such as mosquito cells). In certain embodiments, the packaging cell lines and producer cell lines contain an integrated alphavirus structural protein expression cassette.

Within one embodiment, packaging cell lines are provided which, upon introduction of a vector construct, produce alphavirus particles capable of infecting human cells. Within other embodiments, the packaging cell line produces

alphavirus particles in response to one or more factors. Within certain embodiments, an alphavirus inhibitory protein is not produced within the packaging cell line.

Within other aspects, retroviral-derived packaging cell lines are provided which are suitable for packaging and production of an alphavirus vector. Within one embodiment, a retroviral-derived producer cell line suitable for packaging and production of an alphavirus vector is provided, comprising an expression cassette which directs the expression of gag/pol, an expression cassette which directs the expression of env, and alphavirus vector construct containing a retroviral packaging sequence.

Within another aspect, HBV-derived and coronavirus-derived packaging cell lines are provided which are suitable for packaging and production of and alphavirus vector. Within one embodiment, an HBV-derived packaging cell line is provided, comprising an expression cassette which directs the expression of HBV core, preS/S, and P proteins. Within another embodiment, a coronavirus-derived packaging cell line is provided, comprising an expression cassette which directs the expression of coronavirus N, M, and S proteins.

Within another aspect, a VSV-G derived packaging cell is provided which is suitable for packaging and production of an alphavirus vector, comprising a stably integrated expression cassette which directs the expression of VSV-G. Within a further embodiment, such packaging cell lines comprise a stably integrated expression cassette which directs the expression of one or more alphavirus structural proteins.

Within yet other aspects, producer cell lines are provided based upon the above packaging cell lines. Within one embodiment, such producer cell lines produce recombinant alphavirus particles in response to a differentiation state of the producer cell line. Within other embodiments, such producer cell lines produce recombinant alphavirus particles in response to one or more factors.

As utilized with the context of the present invention, alphavirus producer cell line refers to a cell line which is capable of producing recombinant alphavirus particles. The producer cell line should include an integrated alphavirus structural protein expression cassette capable of directing the expression of alphavirus structural protein(s), and also, an alphavirus vector construct. Preferably, the alphavirus vector construct is a cDNA vector construct. More preferably, the alphavirus vector construct is an integrated cDNA vector construct. When the alphavirus vector construct is an integrated cDNA vector construct, it may, in some instances, function only in response to one or more factors, or the differentiation state of the alphavirus producer cell line.

In still yet another aspect, the present invention provides alphavirus particles which, upon introduction into a BHK cell, produces an infected cell which is viable at least 24 hours and as much as 48, 72, or 96 hours, or 1 week after infection. Also provided are mammalian cells which contain such alphavirus particles. In addition, recombinant alphavirus particles capable of infecting human cells are provided.

In another aspect, the present invention provides recombinant alphavirus particles which, upon introduction into a BHK cell, produces an infected cell which is viable at least 24 hours after infection, the particle also carrying a vector construct which directs the expression of at least one antigen or modified form thereof in target cells infected with the alphavirus particle, the antigen or modified form thereof being capable of stimulating an immune response within an animal. In various embodiments, the expressed antigen or

modified form thereof elicits a cell-mediated immune response, preferably an HLA class I-restricted immune response.

In still another aspect, the present invention provides recombinant alphavirus particles which carry a vector capable of directing the expression of a palliative in cells infected with the alphavirus particle, the palliative being capable of inhibiting a function of a pathogenic agent necessary for pathogenicity. In various embodiments, the pathogenic agent is a virus, fungi, protozoa, or bacteria, and the inhibited function is selected from the group consisting of adsorption, replication, gene expression, assembly, and exit of the pathogenic agent from infected cells. In other embodiments, the pathogenic agent is a cancerous cell, cancer-promoting growth factor, autoimmune disorder, cardiovascular disorders such as restenosis, osteoporosis and male pattern baldness, and the inhibited function is selected from the group consisting of cell viability and cell replication. In further embodiments, the vector directs the expression of a toxic palliative in infected target cells in response to the presence in such cells of an entity associated with the pathogenic agent; preferably the palliative is capable of selectively inhibiting the expression of a pathogenic gene or inhibiting the activity of a protein produced by the pathogenic agent. In still further embodiments, the palliative comprises an inhibiting peptide specific for viral protease, an antisense RNA complementary to RNA sequences necessary for pathogenicity, a sense RNA complementary to RNA sequences necessary for pathogenicity, or a defective structural protein of a pathogenic agent, such protein being capable of inhibiting assembly of the pathogenic agent.

In yet further embodiments, recombinant alphavirus particles described above direct the expression of a palliative, more particularly, direct the expression of a gene product capable of activating an otherwise inactive precursor into an active inhibitor of the pathogenic agent, for example, the herpes thymidine kinase gene product, a tumor suppressor gene, or a protein that activates a compound with little or no cytotoxicity into a toxic product in the presence of a pathogenic agent, thereby effecting localized therapy to the pathogenic agent. Alternatively, the recombinant alphavirus particle directs the expression of a protein that is toxic upon processing or modification by a protein derived from a pathogenic agent, a reporting product on the surface of target cells infected with the alphavirus and containing the pathogenic agent, or an RNA molecule which functions as an antisense or ribozyme specific for a pathogenic RNA molecule required for pathogens. In certain embodiments, in the alphavirus particles described above, the protein is herpes thymidine kinase or CD4.

In yet further aspects, the present invention provides recombinant alphavirus particles which direct the expression of a gene capable of suppressing one or more elements of the immune system in target cells infected with the alphavirus vector, and an alphavirus particle which directs the expression of a blocking element in cells infected with the alphavirus vector, the blocking element being capable of binding to either a receptor or an agent such that the receptor/agent interaction is blocked.

In further aspects, methods are provided for administering any of the above-described recombinant alphavirus particles or vectors, for a prophylactic or therapeutic effect. For example, within one aspect, the present invention provides methods of stimulating an immune response to an antigen, comprising the step of infecting susceptible target cells with a recombinant alphavirus particle which directs the expression of at least one antigen or modified form thereof in target

cells infected with the alphavirus, the antigen or modified form thereof being capable of stimulating an immune response within an animal. In one embodiment, the target cells are infected in vivo, although within other embodiments the target cells are removed, infected ex vivo, and returned to the animal.

In still further aspects of the present invention, methods of stimulating an immune response to a pathogenic antigen are provided, comprising the step of infecting susceptible target cells with a recombinant alphavirus particle which directs the expression of a modified form of a pathogenic antigen in target cells infected with the alphavirus, the modified antigen being capable of stimulating an immune response within an animal but having reduced pathogenicity relative to the pathogenic antigen.

In even further aspects of the present invention, methods of stimulating an immune response to an antigen are provided, comprising infecting susceptible target cells with a recombinant alphavirus particle which directs the expression of a peptide having multiple epitopes, one or more of the epitopes derived from different proteins.

In yet another aspect of the invention, methods of stimulating an immune response within a warm-blooded animal are provided, comprising infecting susceptible target cells associated with a warm-blooded animal with nucleic acid sequences coding for either individual class I or class II MHC protein, or combinations thereof, and infecting the cells with an alphavirus particle which directs the expression of at least one antigen or modified form thereof in target cells infected with the alphavirus particle, the antigen or modified form thereof being capable of stimulating an immune response within the animal.

In another aspect of the present invention, methods of inhibiting a pathogenic agent are provided, comprising infecting susceptible target cells with an alphavirus particle which directs the expression of a palliative in cells infected with the alphavirus particle, the palliative being capable of inhibiting a function of a pathogenic agent necessary for pathogenicity.

As utilized within the context of the present invention, vector or vector constructs which direct the expression of a heterologous sequence of interest in fact refers to the transcribed vector RNA, which directs the expression of the heterologous sequence of interest. In addition, although "animals" are generally referred to, it should be understood that the present invention may be readily applied to a wide variety of animals (both mammalian and non-mammalian), including for example, humans, chimps, macaques, cows, horses, sheep, dogs, birds, cats, fish, rats, and mice. Further, although alphaviruses such as Sindbis may be specifically described herein, it should be understood that a wide variety of other alphaviruses may also be utilized including, for example, Aura, Venezuelan Equine Encephalitis, Fort Morgan, Ross River, Semliki Forest, and Mayaro.

Within other aspects of the present invention, methods are provided for delivering a heterologous nucleic acid sequence to an animal comprising the steps of administering to the warm-blooded animal a eukaryotic layered vector initiation system as described above. Within certain embodiments, the eukaryotic layered vector initiation system may be introduced into the target cells directly as a DNA molecule by physical means, as a complex with various liposome formulations, or as a DNA-ligand complex including the vector molecule (e.g., along with a polycation compound such as polylysine, a receptor specific ligand, or a psoralen inactivated virus such as Sendai or Adenovirus).

Within yet other aspects of the invention, *ex vivo* cells are infected with any of the above-described recombinant alphaviruses are provided. Within yet other aspects, recombinant alphavirus particles are provided which are resistant to inactivation in serum. As utilized herein, recombinant alphavirus particles are considered to be resistant to inactivation in serum if the ratio of surviving particles to input/starting particles in a complement inactivation assay is greater in a statistically significant manner, preferably at least 5-fold, and as much as 10- to 20-fold, as compared to a reference sample produced in BHK cells. Within further aspects, pharmaceutical compositions are provided comprising any of the above-described vectors, or recombinant alphavirus particles, in combination with a physiologically acceptable carrier or diluent.

In yet another aspect of the invention, the eukaryotic layered vector initiation systems provided enable new methods for large scale recombinant protein expression.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (e.g., plasmids, etc.). These references are incorporated herein by reference in their entirety.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration of Sindbis virus genomic organization.

FIG. 2 is an illustration which depicts a method for amplification of a Sindbis RNA genome by RT-PCR.

FIGS. 3A-H set forth the sequence of a representative Eukaryotic Layered Vector Initiation System derived from Sindbis (SEQ. ID NO. 1).

FIG. 4 is a schematic illustration of a Sindbis Basic Vector and a Sindbis-luciferase Vector.

FIG. 5 is an illustration of Sindbis Helper Vector Construction.

FIG. 6 is a graph which illustrates expression and rescue of a Sindbis-luciferase Vector.

FIG. 7 is an illustration of one method for modifying a Sindbis junction region (SEQ ID No:1, positions 7579-7602).

FIG. 8 is a schematic illustration of a representative embodiment of a Eukaryotic Layered Vector Initiation System.

FIG. 9 is a graph which shows a time course for luciferase expression from ELVIS-LUC and SINBV-LUC vectors.

FIG. 10 is a bar graph which depicts the level of ELVIS vector reporter gene expression compared to several different vector constructs.

FIG. 11 is a schematic illustration of Sindbis Packaging Expression Cassettes.

FIG. 12 is a bar graph which shows SIN-luc vector packaging by representative packaging cell lines.

FIG. 13 is a bar graph which shows SIN-luc vector packaging by PCL clone #18 over time.

FIG. 14 is a bar graph which depicts the level of expression by several different luciferase vectors in BHK cells and undifferentiated F9 cells.

FIG. 15 is a schematic illustration of how Astroviruses or other heterologous viruses may be used to express Sindbis structural proteins.

FIG. 16A is a bar graph which shows Sindbis BV-HBe expression and packaging in BHK cells (lysate).

FIG. 16B is a bar graph which shows Sindbis BV-HBe expression and packaging in BHK cells (supernatant).

FIG. 17 is a bar graph which shows Sindbis BV-HB core expression and packaging in BHK cells.

FIG. 18 is a bar graph which shows a comparison of HB core expressed from Sindbis and RETROVECTORS™.

FIG. 19 is a bar graph which shows ELVIS-HBe vector expression in BHK cells.

FIGS. 20A-D are a schematic illustration of several representative mechanisms for activating a disabled viral junction region by "RNA loop-out."

FIG. 21 is a western blot demonstrating expression of capsid protein after transfection with pMAM/C, selection in HAT media, and induction with dexamethasone.

FIGS. 22A-22B depict a bar graph which demonstrates the level of expression of luciferase in BHK cells transfected with ELVIS-LUC vector, and various modifications thereof.

FIG. 23 is a bar graph which demonstrates the level of luciferase or  $\beta$ -galactosidase expression in BHK cells transfected with ELVIS expression vectors, co-transfected with ELVIS expression and helper vectors, or transduced with packaged ELVIS expression vectors.

FIG. 24 depicts a photomicrograph of a ELVIS- $\beta$ -gal injected rat muscle at three days post inoculation. A transverse cryosection from gastrocnemius muscle injected with 50  $\mu$ g of ELVIS- $\beta$ -gal contained in PBS is shown. Four blue stained transverse fibers are evident.

#### DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Alphavirus vector construct" refers to an assembly which is capable of directing the expression of a sequence(s) or gene(s) of interest. The vector construct should include a 5' sequence which is capable of initiating transcription of an alphavirus, as well as sequence(s) which, when expressed, code for biologically active alphavirus non-structural proteins (e.g., NSP1, NSP2, NSP3, and NSP4), and an alphavirus RNA polymerase recognition sequence. In addition, the vector construct should include a viral junction region which may, in certain embodiments, be modified in order to prevent, increase, or reduce viral transcription of the subgenomic fragment, and an alphavirus RNA polymerase recognition sequence. The vector may also include nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, a 5' promoter which is capable of initiating the synthesis of viral RNA *in vitro* from cDNA, as well as one or more restriction sites, and a polyadenylation sequence.

"Alphavirus cDNA vector construct" refers to an assembly which is capable of directing the expression of a sequence(s) or gene(s) of interest. The vector construct should include a 5' sequence which is capable of initiating transcription of an alphavirus, as well as sequence(s) which, when expressed, code for biologically active alphavirus non-structural proteins (e.g., NSP1, NSP2, NSP3, and NSP4), and an alphavirus RNA polymerase recognition sequence. In addition, the vector construct should include a 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA, a viral junction region which may, in certain embodiments, be modified in order to prevent, increase, or reduce viral transcription of the subgenomic fragment, an alphavirus RNA polymerase recognition



sequence, and a 3' sequence which controls transcription termination. The vector may also include nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, splice recognition sequences, a catalytic ribozyme processing sequence, as well as a polyadenylation sequence.

"Expression cassette" refers to a recombinantly produced nucleic acid molecule which is capable of directing the expression of one or more proteins. The expression cassette must include a promoter capable of directing the expression of said proteins, and a sequence encoding one or more proteins, said proteins preferably comprising alphavirus structural protein(s). Optionally, the expression cassette may include transcription termination, splice recognition, and polyadenylation addition sites. Preferred promoters include the CMV, MMTV, MoMLV, and adenovirus VA1RNA promoters. In addition, the expression cassette may contain selectable markers such as Neo, SV2 Neo, hygromycin, phleomycin, histidinol, and DHFR.

"Alphavirus producer cell line" refers to a cell line which is capable of producing recombinant alphavirus particles. The producer cell line should include an integrated alphavirus structural protein expression cassette capable of directing the expression of alphavirus structural protein(s), and also, an alphavirus vector construct. Preferably, the alphavirus vector construct is a cDNA vector construct. More preferably, the alphavirus vector construct is an integrated cDNA vector construct. When the alphavirus vector construct is an integrated cDNA vector construct, it may, in some instances, function only in response to one or more factors, or the differentiation state of the alphavirus producer cell line.

"Recombinant alphavirus particle" refers to a capsid which contains an alphavirus vector construct. Preferably, the capsid is an alphavirus capsid and is contained within a lipid bilayer, such as a cell membrane, in which viral-encoded proteins are embedded. In some instances, the alphavirus vector construct may be contained in a capsid derived from viruses other than alphaviruses (for example, retroviruses, coronaviruses, and hepatitis B virus). A variety of alphavirus vectors may be contained within the recombinant alphavirus particle, including the alphavirus vector constructs of the present invention.

#### A. SOURCES OF ALPHAVIRUS

As noted above, the present invention provides alphavirus vector constructs, alphavirus particles containing such constructs, as well as methods for utilizing such vector constructs and particles. Briefly, sequences encoding wild-type alphavirus suitable for use in preparing the above-described vector constructs and particles may be readily obtained given the disclosure provided herein from naturally-occurring sources, or from depositories (e.g., the American Type Culture Collection, Rockville, Md.).

Representative examples of suitable alphaviruses include Aura (ATCC VR-368), Bebaru virus (ATCC VR-600, ATCC VR-1240), Cabassou (ATCC VR-922), Chikungunya virus (ATCC VR-64, ATCC VR-1241), Eastern equine encephalomyelitis virus (ATCC VR-65, ATCC VR-1242), Fort Morgan (ATCC VR-924), Getah virus (ATCC VR-369, ATCC VR-1243), Kyzylagach (ATCC VR-927), Mayaro (ATCC VR-66), Mayaro virus (ATCC VR-1277), Middleburg (ATCC VR-370), Mucambo virus (ATCC VR-580, ATCC VR-1244), Ndumu (ATCC VR-371), Pixuna virus (ATCC VR-372, ATCC VR-1245), Ross River virus (ATCC VR-373, ATCC VR-1246), Semliki Forest (ATCC VR-67, ATCC VR-1247), Sindbis virus (ATCC VR-68, ATCC VR-1248), Tonate (ATCC VR-925), Trinita (ATCC

VR-469), Una (ATCC VR-374), Venezuelan equine encephalomyelitis (ATCC VR-69), Venezuelan equine encephalomyelitis virus (ATCC VR-923, ATCC VR-1250, ATCC VR-1249, ATCC VR-532), Western equine encephalomyelitis (ATCC VR-70, ATCC VR-1251, ATCC VR-622, ATCC VR-1252), Whataroa (ATCC VR-926), and Y-62-33 (ATCC VR-375).

#### B. SEQUENCES WHICH ENCODE WILD-TYPE SINDBIS VIRUS

Within one particularly preferred aspect of the present invention, the sequences which encode wild-type alphavirus may be obtained from Sindbis virus. In particular, within one embodiment of the invention (and as described in more detail below in Example 1), a Sindbis full-length genomic cDNA clone may be obtained by linking the 5' end of a Sindbis virus cDNA clone to a bacteriophage RNA polymerase promoter, and the 3' end of the cDNA clone to a polyadenosine (poly A) tract of at least 25 nucleotides. In particular, synthesis of the first cDNA strand from the viral RNA template may be accomplished with a 3' oligonucleotide primer having a consecutive sequence comprising an enzyme recognition sequence, a sequence of 25 deoxythymidine nucleotides, and a stretch of approximately 18 nucleotides which is complementary to the viral 3' end, and with a 5' primer containing buffer nucleotides, an enzyme recognition sequence, a bacteriophage promoter, and a sequence complementary to the viral 5' end. The enzyme recognition sites present on each of these primers should be different from each other, and not found in the Sindbis virus. Further, the first nucleotide linked to the 3' end of the bacteriophage RNA polymerase promoter may be the authentic first nucleotide of the RNA virus, or may contain one or more additional non-viral nucleotides. RNA transcribed in vitro from the viral cDNA clone, having the construction described above and linearized by digestion with the unique dT: dA 3' distal restriction enzyme will, after introduction into the appropriate eukaryotic cell, initiate the same infection cycle which is characteristic of infection by the wild-type virus from which the cDNA was cloned. This viral cDNA clone, which yields RNA able to initiate infection after in vitro transcription, is referred to below as an "infectious cDNA clone."

#### C. PRODUCTION OF RECOMBINANT ALPHAVIRUS VECTOR CONSTRUCTS WITH INACTIVATED VIRAL JUNCTION REGIONS

An infectious cDNA clone prepared as described above (or utilizing sequences encoding an alphavirus obtained from other sources) may be readily utilized to prepare alphavirus vector constructs of the present invention. Briefly, within one aspect of the present invention, recombinant alphavirus vector constructs are provided, comprising a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus nonstructural proteins, a viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, and an alphavirus RNA polymerase recognition sequence. As will be discussed in greater detail below, alphavirus vector constructs which have inactivated viral junction regions do not transcribe the subgenomic fragment, making them suitable for a wide variety of applications.

##### 1. RNA POLYMERASE PROMOTER

As noted above, within certain embodiments of the invention alphavirus vector constructs are provided which contain a 5' promoter which is capable of initiating the synthesis of viral RNA in vitro from cDNA. Particularly, preferred 5' promoters include both eukaryotic and prokaryotic

promoters, such as, for example, the  $\beta$ -galactosidase promoter, trpE promoter, lacZ promoter, T7 promoter, T3 promoter, SP6 promoter, SV40 promoter, CMV promoter, and MoMLV LTR.

## 2. SEQUENCES WHICH INITIATE TRANSCRIPTION

As noted above, within preferred embodiments the alphavirus vector constructs of the present invention contain a 5' sequence which is capable of initiating transcription of an alphavirus. Representative examples of such sequences include nucleotides 1-60, and to a lesser extent nucleotides 150-210, of the wild-type Sindbis virus (see FIG. 3), nucleotides 10-75 for tRNA Asparagine (Schlesinger et al., U.S. Pat. No. 5,091,309), and 5' sequences from other Togaviruses which initiate transcription.

## 3. ALPHAVIRUS NONSTRUCTURAL PROTEINS

Alphavirus vector constructs of the present invention should also contain sequences which encode alphavirus nonstructural proteins (NSPs). As an example, for Sindbis virus there are four nonstructural proteins, NSP1, NSP2, NSP3 and NSP4, which encode proteins that enable the virus to self-replicate. Nonstructural proteins 1 through 3 (NSP1-NSP3) are, within one embodiment of the invention, encoded by nucleotides 60 to 5750 of the wild-type Sindbis virus (see FIG. 3). These proteins are produced as a polypeptide and later cleaved into nonstructural proteins NSP1, NSP2, and NSP3. NSP4 is, within one embodiment, encoded by nucleotides 5928 to 7579 (see FIG. 3).

It will be evident to one of ordinary skill in the art that a wide variety of sequences which encode alphavirus nonstructural proteins, in addition to those discussed above, may be utilized in the present invention, and are therefore deemed to fall within the scope of the phrase "Alphavirus Nonstructural Proteins." For example, within one embodiment of the invention, due to the degeneracy of the genetic code, more than one codon may code for a given amino acid. Therefore, a wide variety of nucleic acid sequences which encode alphavirus nonstructural proteins may be generated. Within other embodiments of the invention, a variety of other nonstructural protein derivatives may be made, including for example, various substitutions, insertions, or deletions, the net result of which do not alter the biological activity of the alphavirus nonstructural proteins. Within the context of the present invention, alphavirus nonstructural proteins are deemed to be "biologically active" in toto if they promote the self-replication of the vector construct. Self-replication, which refers to replication of viral nucleic acids and not the production of infectious virus, may be readily determined by metabolic labelling or RNase protection assays performed over a course of time. Methods for making such derivatives may be readily accomplished by one of ordinary skill in the art given the disclosure provided herein (see also, *Molecular Cloning: A Laboratory Manual* (2d. ed.), Cold Spring Harbor Laboratory Press).

## 4. VIRAL JUNCTION REGIONS

Within this aspect of the invention, the alphavirus vector constructs may also include a viral junction region which has been inactivated, such that viral transcription of the subgenomic fragment is prevented. Briefly, the alphavirus viral junction region normally controls transcription initiation of the subgenomic mRNA. In the case of the Sindbis virus, the normal viral junction region typically begins at approximately nucleotide number 7579 and continues through at least nucleotide number 7612 (and possibly beyond). At a minimum, nucleotides 7579 to 7602 (5'-ATC TCT ACG GTG GTC CTAAAT AGT -SEQ. ID NO. 2) are believed necessary for transcription of the subgenomic fragment. This region (nucleotides 7579 to 7602) is hereinafter referred to as the "minimal junction region core."

Within preferred embodiments of the invention (and as described in more detail below), the viral junction region is inactivated in order to prevent viral transcription of the subgenomic fragment. As utilized within the context of the present invention, "inactivated" means that the fragment corresponding to the initiation point of the subgenomic fragment, as measured by a RNase protection assay, is not detected. (Representative assays are described by Melton et al., *Nuc. Acids Res.* 12:7035-7056, 1984; Calzon et al., *Methods in Enz.* 152:611-632, 1987; and Kekule et al., *Nature* 343:457-461, 1990.)

Within one embodiment of the invention, the viral junction region is inactivated by truncating the viral junction region at nucleotide 7597 (i.e., the viral junction region will then consist of the sequence as shown in FIG. 3, from nucleotide 7579 to nucleotide 7597). This truncation prevents transcription of the subgenomic fragment, and additionally permits synthesis of the complete NSP4 region (which is encoded by nucleotides 5928 to 7579).

As will be evident to one of ordinary skill in the art given the disclosure provided herein, a wide variety of other deletions, substitutions or insertions may also be made in order to inactivate the viral junction region. For example, within other embodiments of the invention the viral junction region may be further truncated into the region which encodes NSP4, thereby preventing viral transcription from the subgenomic fragment while retaining the biological activity of NSP4. Alternatively, within other embodiments, due to the redundancy of the genetic code, nucleotide substitutions may be made in the sequence encoding NSP4, the net effect of which does not alter the biological activity of NSP4 yet, nevertheless, prevents transcription of the subgenomic fragment.

## 5. ALPHAVIRUS RNA POLYMERASE RECOGNITION SEQUENCE, AND POLY-A TAIL

As noted above, alphavirus vector constructs of the present invention should also include an alphavirus RNA polymerase recognition sequence (also termed "alphavirus replicase recognition sequence"). Briefly, the alphavirus RNA polymerase recognition sequence provides a recognition site at which the virus begins replication by synthesis of the negative strand. A wide variety of sequences may be utilized as an alphavirus RNA polymerase recognition sequence. For example, within one embodiment, Sindbis vector constructs of the present invention include a Sindbis polymerase recognition sequence which is encoded by nucleotides 11,647 to 11,703 (see FIG. 3). Within other embodiments, the Sindbis polymerase recognition is truncated to the smallest region which can still function as a recognition sequence (e.g., nucleotides 11,684 to 11,703 of FIG. 3).

Within preferred embodiments of the invention, the vector construct may additionally contain a polyA tail. Briefly, the polyA tail may be of any size which is sufficient to promote stability in the cytoplasm, thereby increasing the efficiency of initiating the viral life cycle. Within various embodiments of the invention, the polyA tail comprises at least 10 adenosine nucleotides, and most preferably, at least 25 adenosine nucleotides.

## D. OTHER ALPHAVIRUS VECTOR CONSTRUCTS

In addition to the vector constructs which are generally described above, a wide variety of other alphavirus vector constructs may also be prepared utilizing the disclosure provided herein.

### 1. MODIFIED VIRAL JUNCTION REGIONS

As noted above, the present invention provides viral junction regions which have been modified from the wild-

type sequence. Within the context of the present invention, modified viral junction regions should be understood to include junction regions which have wild-type activity, but a non-wild-type sequence, as well as junction regions with increased, decreased, or no activity. For example, within one aspect of the invention, alphavirus vector constructs are provided wherein the viral junction region has been modified, such that viral transcription of the subgenomic fragment is reduced. Briefly, infection of cells with wild-type alphavirus normally results in cell death as a result of abundant viral transcription of the subgenomic fragment initiated from the viral junction region. This large abundance of RNA molecules can overwhelm the transcriptional machinery of the infected cell, ultimately resulting in death of the cell. In applications where it is desired that infection of a target cell should result in a therapeutic effect (e.g., strand scission of a target nucleic acid or prolonged expression of a heterologous protein) rather than cell death, several modifications to the alphavirus vector construct (in addition to inactivating the vector construct, as described above) may be made in order to reduce the level of viral transcription of the subgenomic fragment, and thereby prolong the life of the vector infected target cell. Within the context of the present invention, viral transcription of the subgenomic fragment is considered to be "reduced" if it produces less subgenomic fragment than a standard wild-type alphavirus (e.g., Sindbis virus ATCC No. VR-1248) as determined by a RNase protection assay.

Viral junction regions may be modified by a variety of methods in order to reduce the level of viral transcription of the subgenomic fragment. For example, within one embodiment of the invention, due to the redundancy of the genetic code nucleotide substitutions may be made in the viral junction region 7579 to 7597, the net effect of which does not alter the amino acid sequence NSP4 (or, within other embodiments, the biological activity of NSP4), and yet reduces the level of viral transcription of the subgenomic fragment. If the modified vector construct includes nucleotides beyond 7597 (e.g., to 7602 or 7612), further nucleotide substitutions may likewise be made, although, since NSP4 terminates at 7597, such substitutions need not be based upon genetic redundancy. Representative examples of modified viral junction regions are described in more detail below in Example 3.

## 2. TANDEM VIRAL JUNCTION REGIONS

Within other aspects of the invention, alphavirus vector constructs are provided, which comprise a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-structural proteins, a first viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, a second viral junction region which is active, or which has been modified such that viral transcription of the subgenomic fragment is reduced, and an alphavirus RNA polymerase recognition sequence. Such vector constructs are referred to as "tandem" vector constructs because they comprise a first inactivated (or "disabled") viral junction region, as well as a second modified ("synthetic") or unmodified viral junction region. Within preferred embodiments of the invention, the inactivated junction region is followed directly by the second viral junction region.

In applications where a low level of subgenomic transcription is required, a minimal junction region core may be inserted downstream in tandem to the inactivated junction region. In order to gradually increase the level of subgenomic transcription for the desired effect, sequences corre-

sponding to the entire junction region may be added to the tandem junction region, in increments.

## 3. THE ADENOVIRUS E3 GENE

Within another aspect of the invention, an adenovirus E3 gene is inserted into a tandem vector construct following the second viral junction region, in order to down-regulate HLA expression in alphavirus infected cells. Briefly, within various embodiments of the invention, repeated inoculations of a gene therapeutic into the same individual is desirable. However, repeated inoculations of alphaviruses such as the Sindbis virus may lead to the development of specific antibodies or cell-mediated immune response against Sindbis viral nonstructural proteins (NSPs). Thus, it may be necessary to mitigate the host immune response targeted to vector-specific proteins in order to administer repeated doses to the same individual.

Therefore, within one embodiment of the invention, products of the Adenovirus type 2 early region gene 3 are utilized in order to down-regulate the expression of integral histocompatibility antigens expressed on the surface of infected cells. Briefly, the E3 19,000 dalton (E3/19K) protein binds to, and forms a molecular complex with, class I H-2/HLA antigens in the endoplasmic reticulum, preventing terminal glycosylation pathways necessary for the full maturation and subsequent transport of the class I H-2/HLA antigens to the cell membrane. In target cells infected with an alphavirus vector encoding the Ad 2 E3 protein, co-expression of the viral nonstructural proteins in the context of class I antigens will not occur. Thus, it is possible to administer repeated doses of an alphavirus vector which expresses the Ad 2 E3 protein as a component of its therapeutic palliative to the same individual. A representative example of the use of the Adenovirus E3 gene is set forth in more detail below in Example 4A.

## 4. THE CMV H301 GENE

Other methods may also be utilized in order to mitigate a host's immune response against viral NSPs. For example, within another aspect of the invention, the human cytomegalovirus ("HCMV") H301 gene is cloned into an alphavirus vector construct, preferably immediately following the second viral junction region in a tandem vector, in order to inhibit host CTL response directed against viral specific proteins expressed in vector infected cells.

Briefly, 2-Microglobulin (2 m) protein binds to the 1, 2 and 3 domains of the alpha-chain of the class I major histocompatibility molecules of higher eukaryotes. Preventing the interaction between 2 m and MHC class I products renders infected cells unrecognizable by cytotoxic T cells. Therefore, as described in greater detail below in Example 4B, expression of the HCMV H301 gene product as a component of a therapeutic palliative may be utilized in order to mitigate the host immune response to viral NSP.

## 5. NONALPHAVIRUS PACKAGING SEQUENCE

Within another aspect of the invention, a packaging sequence derived from a virus other than an alphavirus (for example, retrovirus, coronavirus, hepatitis B virus) is inserted into a tandem vector and positioned between the first (inactivated) viral junction region and the second, modified viral junction region. Briefly, nonalphavirus packaging sequences signal the packaging of an RNA genome into a virus particle corresponding to the source of the packaging sequence. For example, and as described in more detail below, a retroviral packaging sequence may be utilized in order to package an alphavirus vector into a retroviral particle using a retroviral packaging cell line. This is performed in order to increase the efficiency of alphavirus vector transfer into an alphavirus packaging cell line, or to alter the cell or tissue tropism of the alphavirus vector.

## 6. EXPRESSION OF MULTIPLE HETEROLOGOUS GENES

The genomic length and subgenomic length of mRNAs transcribed in wild-type alphavirus infected cells are polycistronic, coding for, respectively, the viral four non-structural proteins (NSPs) and four structural proteins (SPs). The genomic and subgenomic mRNAs are translated as polyproteins, and processing into the individual nonstructural and structural proteins is accomplished by post-translational proteolytic cleavage, catalyzed by viral encoded NSP- and SP- specific proteases, as well as cellular proteases.

In certain applications of the alphavirus vectors described herein, the expression of more than one heterologous gene is desired. For example, in order to treat metabolic disorders such as Gaucher's syndrome, multiple administrations of alphavirus vectors or particles may be required, since duration of the therapeutic palliative may be limited. Therefore, with certain embodiments of the invention it may be desirable to co-express in a target cell the Ad 2 E3 gene (see Example 4), along with a therapeutic palliative, such as the glucocerebrosidase gene (see Example 17). In wild-type virus, however, the structural protein ("SP") polycistronic message is translated into a single polyprotein which is subsequently processed into individual proteins by cleavage with SP-encoded proteases. Thus, expression of multiple heterologous genes from a polycistronic message requires a mechanism different from the wild-type virus, since the SP protease gene, or the peptides recognized for cleavage, are not present in the replacement region of the alphavirus vectors.

Therefore, within one embodiment of the invention alphavirus vectors may be constructed by placing appropriate signals either ribosome readthrough or internal ribosome entry between cistrons. One such representative method of expressing multiple heterologous genes is set forth below in Example 5.

In yet another embodiment of the invention, the placement of signals promoting either ribosome readthrough or internal ribosome entry immediately downstream of the disabled junction region vector pKSSINBVdIJR is described (see Examples 3 and 5). In this vector configuration, synthesis of subgenomic message cannot occur; however, the heterologous proteins are expressed from genomic length mRNA by either ribosomal readthrough (scanning) or internal ribosome entry. Relative to wild-type, the low level of viral transcription with this alphavirus vector would prolong the life of the infected target cell.

In still another embodiment of the invention, placement of signals promoting either ribosome readthrough or internal ribosome entry immediately downstream of the pKSSINBVdIJRsir or pKSSINBV vectors is described. Briefly, since synthesis of subgenomic mRNA occurs in cells infected with the pKSSINBVdIJRsir and pKSSINBV vectors, placement of either a ribosome readthrough sequence or an internal ribosome entry sequence between the two heterologous genes permits translation of both proteins encoded by the subgenomic mRNA polycistronic message. Further, additional heterologous genes can be placed in the subgenomic mRNA region, provided that a suitable translation initiation signal resides at the 5' end of the translational AUG start codon. The number of heterologous gene(s) which can be inserted into the subgenomic mRNA region, as described here, is limited only by the packaging constraints of the vector.

Different sequences which allow either ribosome readthrough, cap-independent translation, or internal ribo-

some entry may be placed into Sindbis vectors pKSSINBVdIJR, pKSSINBV, pKSSINBVdIJRsir, or vectors encompassed by the eukaryotic layered vector initiation system, in the configurations as discussed above. The source of these translation control sequences are the picornaviruses polio and EMCV, the 5' noncoding region of the human immunoglobulin heavy-chain binding protein, and a synthetic sequence of at least 15 bps corresponding in part to the Kozak consensus sequence for efficient translational initiation. Although not described in detail here, these signals which affect translation initiation can also be placed downstream of the junction region and between heterologous genes in all of the modified junction region vectors described in Example 3.

As noted above, the alphavirus cDNA vector construct also includes a 3' sequence which controls transcription termination. A representative example of such a sequence is set forth in more detail in Examples 2 and 3.

## 7. TISSUE SPECIFIC EXPRESSION

Within other aspects of the present invention, alphavirus vector constructs are provided which are capable of expressing a desired heterologous sequence only in a selected tissue. One such representative example is shown in FIG. 20. Briefly, as shown in FIG. 20A, a recombinant alphavirus vector is constructed such that upon introduction of the vector (FIG. 20A) into a target cell, internal inverted repeat sequences which flank the transcriptional control regions (e.g., modified junction region) loop out (see FIG. 20B), thereby preventing viral transcription of subgenomic sequences ("G.O.I.") from the synthetic junction region.

On the other hand, activation of the vector can be attained if the inverted repeats are designed to also hybridize to a specific cellular RNA sequence which is characteristic of a selected tissue or cell type. Such cellular RNA disrupts the disabling stem loop structure, thereby allowing the formation of a more stable secondary stem loop structure (FIGS. 20C and 20D). This secondary stem loop structure allows transcription of the sub-genomic message by placing the junction region back into its correct positional configuration.

Full-length alphavirus vectors can also be transcribed using the secondary stem loop structure by taking advantage of the ability of the viral polymerase to switch templates during synthesis of the negative strand using a strand hopping mechanism termed copy choice (King, *RNA genetics* II, CRC Press, Inc., Boca Raton Fla., Domingo et al. (ed.), pp. 150-185, 1988). Once a single successful round of transcription has occurred, the resulting RNA transcript does not contain inverted repeats because they are deleted as a result of the polymerase copy choice event. This newly synthesized RNA molecule now functions as the primary RNA vector transcript which will transcribe and express as any other non-disabled genomic alphavirus vector previously described. In this RNA vector configuration, tissue or cell-specific activation of the disabled Sindbis vector can be achieved if specific RNA sequences, present only in the targeted cell or tissue types, are used in the design of the inverted repeats. In this fashion alphaviruses such as Sindbis can be engineered to be tissue-specific expression vectors using similar inverted sequences described above.

Using this vector system to achieve tissue specific expression enables a therapeutic alphavirus vector or particle to be delivered systemically into a patient. If the vector should infect a cell which does not express the appropriate RNA species, the vector will only be capable of expressing nonstructural proteins and not the gene of interest. Eventually, the vector will be harmlessly degraded.

Use of the above-described vectors enables virtual tissue-specific expression possible for a variety of therapeutic

applications, including for example, targeting vectors for the treatment for various types of cancers. This rationale relies on specific expression of tumor-specific markers such as the carcinoembryonic tumor specific antigen (CEA) and the alpha-fetoprotein tumor marker. Briefly, utilizing such tumor-specific RNA to target specific tumors allows for the tumor-specific expression of toxic molecules, lymphokines or pro-drugs discussed below. Such methods may be utilized for a wide variety of tumors, including for example, colorectal, lung, breast, ovary, bladder and prostate cancers because all these tumors express the CEA. One representative illustration of vectors suitable for use within this aspect of the present invention is set forth in more detail below in Example 16.

Briefly, CEA was one of the first tumor-specific markers to be described, along with the alpha-fetoprotein tumor marker. CEA is a normal glycoprotein in the embryonic tissue of the gut, pancreas and liver during the first two trimesters of fetal development (*Pathologic Basis of Disease*, 3rd edition 1984, Robbins et al. (eds.)). Previously, CEA was believed to be specific for adenocarcinomas of the colon, however, with the subsequent development of more sensitive radioimmunoassays it became apparent that CEA was presented in the plasma with many endodermally derived cancers, particularly pancreatic, gastric and bronchogenic.

Within related aspects of the present invention, alphavirus cell-specific expression vectors may be constructed to express viral antigens, ribozyme, antisense sequences or immunostimulatory factors such as gamma-interferon ( $\gamma$ -IFN), IL-2 or IL-5 for the targeted treatment of virus infected cell types. In particular, in order to target alphavirus vectors to specific foreign organism or pathogen-infected cells, inverted repeats of the alphavirus vector may be selected to hybridize to any pathogen-specific RNA, for instance target cells infected by pathogens such as HIV, CMV, HBV, HPV and HSV.

Within yet other aspects of the invention, specific organ tissues may be targeted for the treatment of tissue-specific metabolic diseases utilizing gene replacement therapies. For example, the liver is an important target tissue because it is responsible for many of the body's metabolic functions and is associated with many metabolic genetic disorders. Such diseases include many of the glycogen storage diseases, phenylketonuria, Gaucher's disease and familial hypercholesterolemia. Presently there are many liver-specific enzymes and markers which have been sequenced which may be used to engineer appropriate inverted repeats for alphavirus vectors. Such liver-specific cDNAs include sequences encoding for S-adenosylmethionine synthetase (Horikawa et al., *Biochem. Int.* 25:81, 1991); lecithin: cholesterolacyl transferase (Rogne et al., *Biochem. Biophys. Res. Commun.* 148:161, 1987); as well as other liver-specific cDNAs (Chin et al., *Ann. N.Y. Acad. Sci.* 478:120, 1986). Such a liver-specific alphavirus vector could be used to deliver the low density lipoprotein receptor (Yamamoto et al., *Cell* 39:27, 1984) to liver cells for the treatment of familial hypercholesterolemia (Wilson et al., *Mol. Biol. Med.* 7:223, 1990).

#### E. HETEROLOGOUS SEQUENCES

As noted above, a wide variety of nucleotide sequences may be carried by the alphavirus vector constructs of the present invention. Preferably, the nucleotide sequences should be of a size sufficient to allow production of viable virus. Within the context of the present invention, the production of any measurable titer, for example, by plaque assay, luciferase assay, or  $\beta$ -galactosidase assay, of infec-

tious virus on appropriate susceptible monolayers, is considered to be "production of viable virus." This may be, at a minimum, an alphavirus vector construct which does not contain any additional heterologous sequence. However, within other embodiments, the vector construct may contain additional heterologous or foreign sequences. Within preferred embodiments, the heterologous sequence will comprise a heterologous sequence of at least about 100 bases, 2 kb, 3.5 kb, 5 kb, 7 kb, or even a heterologous sequence of at least about 8 kb.

As will be evident to one of ordinary skill in the art given the disclosure provided herein, the efficiency of packaging and hence, viral titer, is to some degree dependent upon the size of the sequence to be packaged. Thus, in order to increase the efficiency of packaging and the production of viable virus, additional non-coding sequences may be added to the vector construct. Moreover, within certain embodiments of the invention it may be desired to increase or decrease viral titer. This increase or decrease may be accomplished by increasing or decreasing the size of the heterologous sequence, and hence the efficiency of packaging.

A wide variety of heterologous sequences may be included in the vector construct, including for example sequences which encode palliatives such as lymphokines, toxins, prodrugs, antigens which stimulate an immune response, ribozymes, and proteins which assist or inhibit an immune response, as well as antisense sequences (or sense sequences for "antisense applications"). As noted above, within various embodiments of the invention the alphavirus vector constructs provided herein may contain (and express, within certain embodiments) two or more heterologous sequences.

#### 1. LYMPHOKINES

Within one embodiment of the invention, the heterologous sequence encodes a lymphokine. Briefly, lymphokines act to proliferate, activate, or differentiate immune effector cells. Representative examples of lymphokines include gamma interferon, tumor necrosis factor, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, GM-CSF, CSF-1 and G-CSF.

Within related embodiments of the invention, the heterologous sequence encodes an immunomodulatory cofactor. Briefly, as utilized within the context of the present invention, "immunomodulatory cofactor" refers to factors which, when manufactured by one or more of the cells involved in an immune response, or when added exogenously to the cells, causes the immune response to be different in quality or potency from that which would have occurred in the absence of the cofactor. The quality or potency of a response may be measured by a variety of assays known to one of skill in the art including, for example, in vitro assays which measure cellular proliferation (e.g.,  $^3\text{H}$  thymidine uptake), and in vitro cytotoxic assays (e.g., which measure  $^{51}\text{Cr}$  release) (see Warner et al., *AIDS Res. and Human Retroviruses* 7:645-655, 1991).

Representative examples of immunomodulatory co-factors include alpha interferon (Finter et al., *Drugs* 42(5):749-765, 1991; U.S. Pat. No. 4,892,743; U.S. Pat. No. 4,966,843; WO 85/02862; Nagata et al., *Nature* 284:316-320, 1980; Familletti et al., *Methods in Enz.* 78:387-394, 1981; Twu et al., *Proc. Natl. Acad. Sci. USA* 86:2046-2050, 1989; Faktor et al., *Oncogene* 5:867-872, 1990), beta interferon (Seif et al., *J. Virol.* 65:664-671, 1991), gamma interferons (Radford et al., *American Society of Hepatology*:2008-2015, 1991; Watanabe et al., *PNAS* 86:9456-9460, 1989; Gansbacher et al., *Cancer Research* 50:7820-7825, 1990; Maio et al., *Can. Immunol. Immu-*

nother. 30:34-42, 1989; U.S. Pat. Nos. 4,762,791 and 4,727, 138), G-CSF (U.S. Pat. Nos. 4,999,291 and 4,810,643), GM-CSF (WO 85/04188), TNFs (Jayaraman et al., *J. Immunology* 144:942-951, 1990), Interleukin-2 (IL-2) (Karupiah et al., *J. Immunology* 144:290-298, 1990; Weber et al., *J. Exp. Med.* 166:1716-1733, 1987; Gansbacher et al., *J. Exp. Med.* 172:1217-1224, 1990; U.S. Pat. No. 4,738,927), IL-4 (Tepper et al., *Cell* 57:503-512, 1989; Golumbek et al., *Science* 254:713-716, 1991; U.S. Pat. No. 5,017,691), IL-6 (Brakenhof et al., *J. Immunol.* 139:4116-4121, 1987; WO 90/06370), IL-12, IL-15 (Grabstein et al., *Science* 264:965-968, 1994; Genbank-EMBL Accession No. V03099), ICAM-1 (Altman et al., *Nature* 338:512-514, 1989), ICAM-2, LFA-1, LFA-3, MHC class I molecules, MHC class II molecules,  $\alpha_2$ -microglobulin, chaperones, CD3, B7/BB1, MHC linked transporter proteins or analogues thereof.

The choice of which immunomodulatory cofactor to include within a alphavirus vector construct may be based upon known therapeutic effects of the cofactor, or experimentally determined. For example, in chronic hepatitis B infections alpha interferon has been found to be efficacious in compensating a patient's immunological deficit and thereby assisting recovery from the disease. Alternatively, a suitable immunomodulatory cofactor may be experimentally determined. Briefly, blood samples are first taken from patients with a hepatic disease. Peripheral blood lymphocytes (PBLs) are restimulated in vitro with autologous or HLA-matched cells (e.g., EBV transformed cells), and transduced with an alphavirus vector construct which directs the expression of an immunogenic portion of a hepatitis antigen and the immunomodulatory cofactor. Stimulated PBLs are used as effectors in a CTL assay with the HLA-matched transduced cells as targets. An increase in CTL response over that seen in the same assay performed using HLA-matched stimulator and target cells transduced with a vector encoding the antigen alone, indicates a useful immunomodulatory cofactor. Within one embodiment of the invention, the immunomodulatory cofactor gamma interferon is particularly preferred.

Another example of an immunomodulatory cofactor is the B7/BB1 costimulatory factor. Briefly, activation of the full functional activity of T cells requires two signals. One signal is provided by interaction of the antigen-specific T cell receptor with peptides which are bound to major histocompatibility complex (MHC) molecules, and the second signal, referred to as costimulation, is delivered to the T cell by antigen-presenting cells. Briefly, the second signal is required for interleukin-2 (IL-2) production by T cells and appears to involve interaction of the B7/BB1 molecule on antigen-presenting cells with CD28 and CTLA-4 receptors on T lymphocytes (Linsley et al., *J. Exp. Med.*, 173:721-730, 1991a, and *J. Exp. Med.*, 174:561-570, 1991). Within one embodiment of the invention, B7/BB1 may be introduced into tumor cells in order to cause costimulation of CD8<sup>+</sup> T cells, such that the CD8<sup>+</sup> T cells produce enough IL-2 to expand and become fully activated. These CD8<sup>+</sup> T cells can kill tumor cells that are not expressing B7 because costimulation is no longer required for further CTL function. Vectors that express both the costimulatory B7/BB1 factor and, for example, an immunogenic HBV core protein, may be made utilizing methods which are described herein. Cells transduced with these vectors will become more effective antigen-presenting cells. The HBV core-specific CTL response will be augmented from the fully activated CD8<sup>+</sup> T cell via the costimulatory ligand B7/BB1.

## 2. TOXINS

Within another embodiment of the invention, the heterologous sequence encodes a toxin. Briefly, toxins act to directly inhibit the growth of a cell. Representative examples of toxins include ricin (Lamb et al., *Eur. J. Biochem.* 148:265-270, 1985), abrin (Wood et al., *Eur. J. Biochem.* 198:723-732, 1991; Evensen et al., *J. of Biol. Chem.* 266:6848-6852, 1991; Collins et al., *J. of Biol. Chem.* 265:8665-8669, 1990; Chen et al., *Fed. of Eur. Biochem. Soc.* 309:115-118, 1992), diphtheria toxin (Tweten et al., *J. Biol. Chem.* 260:10392-10394, 1985), cholera toxin (Mekalanos et al., *Nature* 306:551-557, 1983; Sanchez and Holmgren, *PNAS* 86:481-485, 1989), gelonin (Stürpe et al., *J. Biol. Chem.* 255:6947-6953, 1980), pokeweed (Irvin, *Pharmac. Ther.* 21:371-387, 1983), antiviral protein (Barbieri et al., *Biochem. J.* 203:55-59, 1982; Irvin et al., *Arch. Biochem. & Biophys.* 200:418-425, 1980; Irvin, *Arch. Biochem. & Biophys.* 169:522-528, 1975), tritin, Shigella toxin (Calderwood et al., *PNAS* 84:4364-4368, 1987; Jackson et al., *Microb. Path.* 2:147-153, 1987), Pseudomonas exotoxin A (Carroll and Collier, *J. Biol. Chem.* 262:8707-8711, 1987), herpes simplex virus thymidine kinase (HSVTK) (Field et al., *J. Gen. Virol.* 49:115-124, 1980), and *E. coli*, guanine phosphoribosyl transferase.

## 3. PRO-DRUGS

Within other embodiments of the invention, the heterologous sequence encodes a "pro-drug". Briefly, as utilized within the context of the present invention, "pro-drug" refers to a gene product that activates a compound with little or no cytotoxicity into a toxic product. Representative examples of such gene products include HSVTK and VZVTK (as well as analogues and derivatives thereof), which selectively monophosphorylate certain purine arabinosides and substituted pyrimidine compounds, converting them to cytotoxic or cytostatic metabolites. More specifically, exposure of the drugs ganciclovir, acyclovir, or any of their analogues (e.g., FIAU, FIAC, DHPG) to HSVTK phosphorylates the drug into its corresponding active nucleotide triphosphate form.

Representative examples of other pro-drugs which may be utilized within the context of the present invention include: *E. coli* guanine phosphoribosyl transferase which converts thioxanthine into toxic thioxanthine monophosphate (Besnard et al., *Mol. Cell. Biol.* 7:4139-4141, 1987); alkaline phosphatase, which will convert inactive phosphorylated compounds such as mitomycin phosphate and doxorubicin-phosphate to toxic dephosphorylated compounds; fungal (e.g., *Fusarium oxysporum*) or bacterial cytosine deaminase, which will convert 5-fluorocytosine to the toxic compound 5-fluorouracil (Mullen, *PNAS* 89:33, 1992); carboxypeptidase G2, which will cleave the glutamic acid from para-N-bis (2-chloroethyl) aminobenzoyl glutamic acid, thereby creating a toxic benzoic acid mustard; and Penicillin-V amidase, which will convert phenoxycetide derivatives of doxorubicin and melphalan to toxic compounds (see generally, Vrudhula et al., *J. of Med. Chem.* 36(7):919-923, 1993; Kern et al., *Canc. Immun. Immunother.* 31(4):202-206, 1990).

## 4. ANTISENSE SEQUENCES

Within another embodiment of the invention, the heterologous sequence is an antisense sequence. Briefly, antisense sequences are designed to bind to RNA transcripts, and thereby prevent cellular synthesis of a particular protein or prevent use of that RNA sequence by the cell. Representative examples of such sequences include antisense thymidine kinase, antisense dihydrofolate reductase (Maher and Dolnick, *Arch. Biochem. & Biophys.* 253:214-220, 1987; Bzik et al., *PNAS* 84:8360-8364, 1987), antisense HER2

(Coussens et al., *Science* 230:1132-1139, 1985), antisense ABL (Fainstein et al., *Oncogene* 4:1477-1481, 1989), antisense Myc (Stanton et al., *Nature* 310:423-425, 1984) and antisense ras, as well as antisense sequences which block any of the enzymes in the nucleotide biosynthetic pathway. In addition, within other embodiments of the invention antisense sequences to interferon and 2 microglobulin may be utilized in order to decrease immune response.

In addition, within a further embodiment of the invention, antisense RNA may be utilized as an anti-tumor agent in order to induce a potent Class I restricted response. Briefly, in addition to binding RNA and thereby preventing translation of a specific mRNA, high levels of specific antisense sequences are believed to induce the increased expression of interferons (including gamma-interferon) due to the formation of large quantities of double-stranded RNA. The increased expression of gamma interferon, in turn, boosts the expression of MHC Class I antigens. Preferred antisense sequences for use in this regard include actin RNA, myosin RNA, and histone RNA. Antisense RNA which forms a mismatch with actin RNA is particularly preferred.

#### 5. RIBOZYMES

Within other aspects of the present invention, alphavirus vectors are provided which produce ribozymes upon infection of a host cell. Briefly, ribozymes are used to cleave specific RNAs and are designed such that it can only affect one specific RNA sequence. Generally, the substrate binding sequence of a ribozyme is between 10 and 20 nucleotides long. The length of this sequence is sufficient to allow a hybridization with target RNA and disassociation of the ribozyme from the cleaved RNA. Representative examples for creating ribozymes include those described in U.S. Pat. Nos. 5,116,742; 5,225,337 and 5,246,921. Particularly preferred ribozymes for use within the present invention include those disclosed in more detail below in the Examples (e.g., Examples 18 and 19).

#### 6. PROTEINS AND OTHER CELLULAR CONSTITUENTS

Within other aspects of the present invention, a wide variety of proteins or other cellular constituents may be carried by the alphavirus vector construct. Representative examples of such proteins include native or altered cellular components, as well as foreign proteins or cellular constituents, found in for example, viruses, bacteria, parasites or fungus.

##### (a) Altered Cellular Components

Within one embodiment, alphavirus vector constructs are provided which direct the expression of an immunogenic, non-tumorigenic, altered cellular component. As utilized herein, the term "immunogenic" refers to altered cellular components which are capable, under the appropriate conditions, of causing an immune response. This response must be cell-mediated, and may also include a humoral response. The term "non-tumorigenic" refers to altered cellular components which will not cause cellular transformation or induce tumor formation in nude mice. The phrase "altered cellular component" refers to proteins and other cellular constituents which are either associated with rendering a cell tumorigenic, or are associated with tumorigenic cells in general, but are not required or essential for rendering the cell tumorigenic.

Before alteration, the cellular components may be essential to normal cell growth and regulation and include, for example, proteins which regulate intracellular protein degradation, transcriptional regulation, cell-cycle control, and cell-cell interaction. After alteration, the cellular components no longer perform their regulatory functions and,

hence, the cell may experience uncontrolled growth. Representative examples of altered cellular components include ras\*, p53\*, Rb\*, altered protein encoded by the Wilms' tumor gene, ubiquitin\*, mucin\*, protein encoded by the DCC, APC, and MCC genes, the breast cancer gene BRCA1\*, as well as receptors or receptor-like structures such as neu, thyroid hormone receptor, platelet derived growth factor (PDGF) receptor, insulin receptor, epidermal growth factor (EGF) receptor, and the colony stimulating factor (CSF) receptor.

Within one embodiment of the present invention, alphavirus vector constructs are provided which direct the expression of a non-tumorigenic, altered ras (ras\*) gene. Briefly, the ras\* gene is an attractive target because it is causally linked to the neoplastic phenotype, and indeed may be necessary for the induction and maintenance of tumorigenesis in a wide variety of distinct cancers, such as pancreatic carcinoma, colon carcinoma and lung adenocarcinoma. In addition, ras\* genes are found in pre-neoplastic tumors and, therefore, immune intervention therapy may be applied prior to detection of a malignant tumor.

Normal ras genes are non-tumorigenic and ubiquitous in all mammals. They are highly conserved in evolution and appear to play an important role in maintenance of the cell cycle and normal growth properties. The normal ras protein is a G-protein which binds GTP and has GTPase activity, and is involved in transmitting signals from the external milieu to the inside of the cell, thereby allowing a cell to respond to its environment. Ras\* genes on the other hand alter the normal growth regulation of neoplastic cells by uncoupling cellular behavior from the environment, thus leading to the uncontrolled proliferation of neoplastic cells. Mutation of the ras gene is believed to be an early event in carcinogenesis (Kumar et al., *Science* 248:1101-1104, 1990) which, if treated early, may prevent tumorigenesis.

Ras\* genes occur in a wide variety of cancers, including for example, pancreatic, colon, and lung adenocarcinomas. The spectrum of mutations occurring in the ras\* genes found in a variety of cancers is quite limited. These mutations alter the GTPase activity of the ras protein by converting the normal on/off switch to a constitutive ON position. Tumorigenic mutations in ras\* occur primarily (in vivo) in only 3 codons: 12, 13 and 61. Codon 12 mutations are the most prevalent in both human and animal tumors.

Table 1 below summarizes known in vivo mutations (codons 12, 13 and 61) which activate human ras, as well as potential mutations which have in vitro transforming activity. Potential mutations with in vitro transforming activity were produced by the systematic substitution of amino acids for the normal codon (e.g., other amino acids were substituted for the normal glycine at position 12). In vitro mutations, while not presently known to occur in humans or animals, may serve as the basis for an anti-cancer immunotherapeutic if they are eventually found to arise in vivo.

TABLE 1

AMINO ACID SUBSTITUTIONS THAT ACTIVATE HUMAN RAS PROTEINS								
Amino Acid	Gly	Gly	Ala	Gln	Glu	Asn	Lys	Asp
Mutant Codon	12	13	59	61	63	116	117	119
In vivo	Val	Asp		Arg				
	Arg	Val		His				
	Asp	Arg		Leu				
	Cys							



TABLE 1-continued

AMINO ACID SUBSTITUTIONS THAT ACTIVATE HUMAN RAS PROTEINS								
In vitro	Ala							
	Ser							
	Phe							
	Ala	Ser	Thr	Val	Lys	His	Glu	His
	Asn			Ala		Ile	Arg	Glu
	Gln			Cys				Ala
	Glu			Asn				Asn
	His			Ile				
	Ile			Met				
	Leu			Thr				
	Lys			Tyr				
	Met			Trp				
	Phe			Phe				
	Ser			Gly				
	Thr							
	Trp							
	Tyr							

Alterations as described above result in the production of proteins containing novel coding sequence(s). The novel proteins encoded by these sequence(s) may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding regions may be utilized to destroy tumorigenic cells containing the altered sequences (ras\*).

Within another embodiment of the present invention, alphavirus vector constructs are provided which direct the expression of an altered p53 (p53\*) gene. Briefly, p53 is a nuclear phosphoprotein which was originally discovered in extracts of transformed cells and thus was initially classified as an oncogene (Linzer and Levine, *Cell* 17:43-52, 1979; Lane and Crawford, *Nature* 278:261-263, 1979). It was later discovered that the original p53 cDNA clones were mutant forms of p53 (Hinds et al., *J. Virol.* 63:739-746, 1989). It now appears that p53 is a tumor suppressor gene which negatively regulates the cell cycle, and that mutation of this gene may lead to tumor formation. Of colon carcinomas that have been studied, 75%-80% show a loss of both p53 alleles, one through deletion and the other through point mutation. Similar mutations are found in lung cancer, and in brain and breast tumors.

The majority of p53 mutations (e.g., p53<sup>\*1</sup>, p53<sup>\*2</sup>, etc.) are clustered between amino acid residues 130 to 290 (see Levine et al., *Nature* 351:453-456, 1991; see also the following references which describe specific mutations in more detail: Baker et al., *Science* 244:217-221, 1989; Nigro et al., *Nature* 342:705-708, 1989 (p53 mutations cluster at four "hot spots" which coincide with the four highly conserved regions of the genes and these mutations are observed in human brain, breast, lung and colon tumors); Vogelstein, *Nature* 348:681-682, 1990; Takahashi et al., *Science* 246:491-494, 1989; Iggo et al., *Lancet* 335:675-679, 1990; James et al., *Proc. Natl. Acad. Sci. USA* 86:2858-2862, 1989; Mackay et al., *Lancet* 11:1384-1385, 1988; Kelman et al., *Blood* 74:2318-2324, 1989; Malkin et al., *Science* 250:1233-1238, 1990; Baker et al., *Cancer Res.* 50:7717-7722, 1991; Chiba et al., *Oncogene* 5:1603-1610, 1990 (pathogenesis of early stage non-small cell lung cancer is associated with somatic mutations in the p53 gene between codons 132 to 283); Prosser et al., *Oncogene* 5:1573-1579, 1990 (mutations in the p53 gene coding for amino acids 126 through 224 were identified in primary breast cancer); Cheng and Hass, *Mol. Cell. Biol.* 10:5502-5509, 1990; Bartek et al., *Oncogene* 5:893-899, 1990; Rodrigues et al., *Proc. Natl. Acad. Sci. USA*

87:7555-7559, 1990; Menon et al., *Proc. Natl. Acad. Sci. USA* 87:5435-5439, 1990; Mulligan et al., *Proc. Natl. Acad. Sci. USA* 87:5863-5867, 1990; and Romano et al., *Oncogene* 4:1483-1488, 1990 (identification of a p53 mutation at codon 156 in human osteosarcoma derived cell line HOS-SL)).

Certain alterations of the p53 gene may be due to certain specific toxins. For example, Bressac et al. (*Nature* 350:429-431, 1991) describes specific G to T mutations in codon 249 in patients affected with hepatocellular carcinoma. One suggested causative agent of this mutation is aflatoxin B<sub>1</sub>, a liver carcinogen which is known to be a food contaminant in Africa.

Four regions of the gene that are particularly affected occur at residues 132-145, 171-179, 239-248, and 272-286. Three "hot spots" which are found within these regions that are of particular interest occur at residues 175, 248 and 273 (Levine et al., *Nature* 351:453-456, 1991). These alterations, as well as others which are described above, result in the production of protein(s) which contain novel coding sequence(s). The novel proteins encoded by these sequences may be used as a marker of tumorigenic cells and an immune response directed against these novel coding regions may be utilized to destroy tumorigenic cells containing the altered sequence (p53\*).

Once a sequence encoding the altered cellular component has been obtained, it is necessary to ensure that the sequence encodes a non-tumorigenic protein. Various assays which assess the tumorigenicity of a particular cellular component are known and may easily be accomplished. Representative assays include a rat fibroblast assay, tumor formation in nude mice or rats, colony formation in soft agar, and preparation of transgenic animals, such as transgenic mice.

Tumor formation in nude mice or rats is a particularly important and sensitive method for determining the tumorigenicity of a particular cellular component. Nude mice lack a functional cellular immune system (i.e., do not possess CTLs), and therefore provide a useful in vivo model in which to test the tumorigenic potential of cells. Normal non-tumorigenic cells do not display uncontrolled growth properties if infected into nude mice. However, transformed cells will rapidly proliferate and generate tumors in nude mice. Briefly, in one embodiment the alphavirus vector construct is administered to syngeneic murine cells, followed by injection into nude mice. The mice are visually examined for a period of 2 to 8 weeks after injection in order to determine tumor growth. The mice may also be sacrificed and autopsied in order to determine whether tumors are present. (Giovanella et al., *J. Natl. Cancer Inst.* 48:1531-1533, 1972; Furesz et al., *Abnormal Cells, New Products and Risk*, Hopps and Petricciani (eds.), Tissue Culture Association, 1985; and Levenbook et al., *J. Biol. Std.* 13:135-141, 1985.)

Tumorigenicity may also be assessed by visualizing colony formation in soft agar (Macpherson and Montagnier, *Vir.* 23:291-294, 1964). Briefly, one property of normal non-tumorigenic cells is "contact inhibition" (i.e., cells will stop proliferating when they touch neighboring cells). If cells are plated in a semi-solid agar support medium, normal cells rapidly become contact inhibited and stop proliferating, whereas tumorigenic cells will continue to proliferate and form colonies in soft agar.

Transgenic animals, such as transgenic mice, may also be utilized to assess the tumorigenicity of an altered cellular component. (Stewart et al., *Cell* 38:627-637, 1984; Quaife et al., *Cell* 48:1023-1034, 1987; and Koike et al., *Proc. Natl. Acad. Sci. USA* 86:5615-5619, 1989.) In transgenic animals,



the gene of interest may be expressed in all tissues of the animal. This dysregulated expression of the transgene may serve as a model for the tumorigenic potential of the newly introduced gene.

If the altered cellular component is associated with making the cell tumorigenic, then it is necessary to make the altered cellular component non-tumorigenic. For example, within one embodiment the sequence or gene of interest which encodes the altered cellular component is truncated in order to render the gene product non-tumorigenic. The gene encoding the altered cellular component may be truncated to a variety of sizes, although it is preferable to retain as much as possible of the altered cellular component. In addition, it is necessary that any truncation leave intact at least some of the immunogenic sequences of the altered cellular component. Alternatively, multiple translational termination codons may be introduced downstream of the immunogenic region. Insertion of termination codons will prematurely terminate protein expression, thus preventing expression of the transforming portion of the protein.

Within one embodiment, the *ras*\* gene is truncated in order to render the *ras*\* protein non-tumorigenic. Briefly, the carboxy-terminal amino acids of *ras*\* functionally allow the protein to attach to the cell membrane. Truncation of these sequences renders the altered cellular component non-tumorigenic. Preferably, the *ras*\* gene is truncated in the purine ring binding site, for example around the sequence which encodes amino acid number 110. The *ras*\* gene sequence may be truncated such that as little as about 20 amino acids (including the altered amino acid(s)) are encoded by the alphavirus vector construct, although preferably, as many amino acids as possible should be expressed (while maintaining non-tumorigenicity).

Within another embodiment, the *p53*\* protein is modified by truncation in order to render the cellular component non-tumorigenic. As noted above, not all mutations of the *p53* protein are tumorigenic, and therefore, not all mutations would have to be truncated. Nevertheless, within a preferred embodiment, *p53*\* is truncated to a sequence which encodes amino acids 100 to 300, thereby including all four major "hot spots."

Other altered cellular components which are oncogenic may also be truncated in order to render them non-tumorigenic. For example, both *neu* and *bcr/ab1* may be truncated in order to render them non-tumorigenic. Non-tumorigenicity may be confirmed by assaying the truncated altered cellular component as described above.

It should be noted, however, that if the altered cellular component is only associated with non-tumorigenic cells in general, and is not required or essential for making the cell tumorigenic, then it is not necessary to render the cellular component non-tumorigenic. Representative examples of such altered cellular components which are not tumorigenic include *Rb*\*, *ubiquitin*\*, and *mucin*\*.

As noted above, in order to generate an appropriate immune response, the altered cellular component must also be immunogenic. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes often possess an immunogenic amphipathic alpha-helix component. In general, however, it is preferable to determine immunogenicity in an assay. Representative assays include an ELISA, which detects the presence of antibodies against the newly introduced vector, as well as assays which test for T helper cells such as gamma-interferon assays, IL-2 production assays, and proliferation assays.

As noted above, within another aspect of the present invention, several different altered cellular components may

be co-expressed in order to form a general anti-cancer therapeutic. Generally, it will be evident to one of ordinary skill in the art that a variety of combinations can be made. Within preferred embodiments, this therapeutic may be targeted to a particular type of cancer. For example, nearly all colon cancers possess mutations in *ras*, *p53*, *DCC* *APC* or *MCC* genes. An alphavirus vector construct which co-expresses a number of these altered cellular components may be administered to a patient with colon cancer in order to treat all possible mutations. This methodology may also be utilized to treat other cancers. Thus, an alphavirus vector construct which co-expresses *mucin*\*, *ras*\*, *neu*, *BRCA1*\* and *p53*\* may be utilized to treat breast cancer.

#### (b) Antigens from foreign organisms or other pathogens

Within other aspects of the present invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of antigens from foreign organisms or other pathogens. Representative examples of such antigens include bacterial antigens (e.g., *E. coli*, streptococcal, staphylococcal, mycobacterial, etc.), fungal antigens, parasitic antigens, and viral antigens (e.g., influenza virus, Human Immunodeficiency Virus ("HIV"), Hepatitis A, B and C Virus ("HAV", "HBV" and "HCV", respectively), Human Papilloma Virus ("HPV"), Epstein-Barr Virus ("EBV"), Herpes Simplex Virus ("HSV"), Hantavirus, HTLV I, HTLV II and Cytomegalovirus ("CMV")). As utilized within the context of the present invention, "immunogenic portion" refers to a portion of the respective antigen which is capable, under the appropriate conditions, of causing an immune response (i.e., cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen. Cell-mediated immune responses may be mediated through Major Histocompatibility Complex ("MHC") class I presentation, MHC Class II presentation, or both.

Within one aspect of the invention, alphavirus vector constructs are provided which direct the expression of immunogenic portions of Hepatitis B antigens. Briefly, the Hepatitis B genome is comprised of circular DNA of about 3.2 kilobases in length and has been well characterized (Tiollais et al., *Science* 213:406-411, 1981; Tiollais et al., *Nature* 317:489-495, 1985; and Ganem and Varmus, *Annu. Rev. Biochem.* 56:651-693, 1987; see also EP 0 278,940, EP 0 241,021, WO 88/10301, and U.S. Pat. Nos. 4,696,898 and 5,024,938, which are hereby incorporated by reference). The Hepatitis B virus presents several different antigens, including among others, three HB "S" antigens (HBsAgs), an HBc antigen (HBcAg), an HBe antigen (HBeAg), and an HBx antigen (HBxAg) (see Blum et al., *TIG* 5(5):154-158, 1989). Briefly, the HBeAg results from proteolytic cleavage of a P22 pre-core intermediate and is secreted from the cell. HBcAg is found in serum as a 17 kD protein. The HBcAg is a protein of 183 amino acids, and the HBxAg is a protein of 145 to 154 amino acids, depending on subtype.

The HBsAgs (designated "large," "middle" and "small") are encoded by three regions of the Hepatitis B genome: S, pre-S2 and pre-S1. The large protein, which has a length varying from 389 to 400 amino acids, is encoded by pre-S1, pre-S2, and S regions, and is found in glycosylated and non-glycosylated forms. The middle protein is 281 amino acids long and is encoded by the pre-S2 and S regions. The small protein is 226 amino acids long and is encoded by the S region. It exists in two forms, glycosylated (GP 27\*) and non-glycosylated (P24\*). If each of these regions are expressed separately, the pre-S1 region will code for a protein of approximately 119 amino acids, the pre-S2 region will code for a protein of approximately 55 amino acids, and the S region will code for a protein of approximately 226 amino acids.

As will be evident to one of ordinary skill in the art, various immunogenic portions of the above-described S antigens may be combined in order to induce an immune response when administered by one of the alphavirus vector constructs described herein. In addition, due to the large immunological variability that is found in different geographic regions for the S open reading frame of HBV, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, epitopes that are found in all human hepatitis B virus S samples are defined as determinant "α". Mutually exclusive subtype determinants, however, have also been identified by two-dimensional double immunodiffusion (Ouchterlony, *Progr. Allergy* 5:1, 1958). These determinants have been designated "d" or "y" and "w" or "r" (LeBouvier, *J. Infect. Dis.* 123:671, 1971; Bancroft et al., *J. Immunol.* 109:842, 1972; and Courouce et al., *Bibl. Haematol.* 42:1-158, 1976). The immunological variability is due to single nucleotide substitutions in two areas of the hepatitis B virus S open reading frame, resulting in the following amino acid changes: (1) exchange of lysine-122 to arginine in the Hepatitis B virus S open reading frame causes a subtype shift from d to y, and (2) exchange of arginine-160 to lysine causes the shift from subtype r to w. In Africans, subtype ayw is predominant, whereas in the U.S. and northern Europe the subtype adw<sub>2</sub> is more abundant (*Molecular Biology of the Hepatitis B Virus*, McLachlan (ed.), CRC Press, 1991). As will be evident to one of ordinary skill in the art, it is generally preferred to construct a vector for administration which is appropriate to the particular hepatitis B virus subtype which is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, preferably, by sequencing the S open reading frame of HBV virus isolated from individuals within that region.

Also presented by HBV are pol ("HBV pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected livers. The polymerase protein consists of at least two domains: the amino terminal domain which encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of HBV pol may be determined utilizing methods described herein (e.g., below and in Example 13), utilizing alphavirus vector constructs described below, and administered in order to generate an immune response within a warm-blooded animal. Similarly, other HBV antigens, such as ORF 5 and ORF 6 (Miller et al., *Hepatology* 9:322-327, 1989) may be expressed utilizing alphavirus vector constructs as described herein. Representative examples of alphavirus vector constructs utilizing ORF 5 and ORF 6 are set forth below in the examples.

As noted above, at least one immunogenic portion of a hepatitis B antigen is incorporated into an alphavirus vector construct. The immunogenic portion(s) which are incorporated into the alphavirus vector construct may be of varying length, although it is generally preferred that the portions be at least 9 amino acids long and may include the entire antigen. Immunogenicity of a particular sequence is often difficult to predict, although T cell epitopes may be predicted utilizing computer algorithms such as TSITES (MedImmune, Maryland), in order to scan coding regions for potential T-helper sites and CTL sites. From this analysis, peptides are synthesized and used as targets in an in vitro cytotoxic assay. Other assays, however, may also be utilized, including, for example, ELISA, which detects the presence

of antibodies against the newly introduced vector, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays and proliferation assays.

Immunogenic portions may also be selected by other methods. For example, the HLA A2.1 transgenic mouse has been shown to be useful as a model for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T cell receptor repertoire recognizes the same antigenic determinants recognized by human T cells. In both systems, the CTL response generated in the HLA A2.1 transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al., *J. Exp. Med.* 173:1007-1015, 1991; Vitiello et al., *Abstract of Molecular Biology of Hepatitis B Virus Symposia*, 1992).

Particularly preferred immunogenic portions for incorporation into alphavirus vector constructs include HBeAg, HBcAg and HBsAg, as described in greater detail below in Example 13.

Additional immunogenic portions of the hepatitis B virus may be obtained by truncating the coding sequence at various locations including, for example, the following sites: Bst UI, Ssp I, Ppu M1, and Msp I (Valenzuela et al., *Nature* 280:815-19, 1979; Valenzuela et al., *Animal Virus Genetics: ICN/UCLA Symp. Mol. Cell Biol.*, 1980, B. N. Fields and R. Jaenisch (eds.), pp. 57-70, New York: Academic). Further methods for determining suitable immunogenic portions as well as methods are also described below in the context of hepatitis C.

As noted above, more than one immunogenic portion may be incorporated into the alphavirus vector construct. For example, an alphavirus vector construct may express (either separately or as one construct) all or immunogenic portions of HBcAg, HBeAg, HBsAg, HBxAg, as well as immunogenic portions of HCV antigens.

#### 7. SOURCES FOR HETEROLOGOUS SEQUENCES

Sequences which encode the above-described proteins may be readily obtained from a variety of sources, including for example, depositories such as the American Type Culture Collection (ATCC, Rockville, Md.), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford, England). Representative examples include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids); BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contain sequences encoding alpha interferon), ATCC Nos. 31902, 31902 and 39517 (which contains sequences encoding beta interferon), ATCC No. 67024 (which contain a sequence which encodes Interleukin-1b); ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2); ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3); ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6).

Sequences which encode altered cellular components as described above may be readily obtained from a variety of sources. For example, plasmids which contain sequences that encode altered cellular products may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Md.), or from commercial sources such as Advanced Biotechnologies (Columbia, Md.). Representative examples of plasmids containing some of the above-described sequences include ATCC No. 41000 (containing a

G to T mutation in the 12th codon of ras), and ATCC No. 41049 (containing a G to A mutation in the 12th codon).

Alternatively, plasmids which encode normal cellular components may also be obtained from depositories such as the ATCC (see, for example, ATCC No. 41001, which contains a sequence which encodes the normal ras protein; ATCC No. 57103, which encodes abl; and ATCC Nos. 59120 or 59121, which encode the bcr locus) and mutated to form the altered cellular component. Methods for mutagenizing particular sites may readily be accomplished using methods known in the art (see Sambrook et al., supra., 15.3 et seq.). In particular, point mutations of normal cellular components such as ras may readily be accomplished by site-directed mutagenesis of the particular codon, for example, codons 12, 13 or 61.

Sequences which encode the above-described viral antigens may likewise be obtained from a variety of sources. For example, molecularly cloned genomes which encode the hepatitis B virus may be obtained from sources such as the American Type Culture Collection (ATCC, Rockville, Md.). For example, ATCC No. 45020 contains the total genomic DNA of hepatitis B (extracted from purified Dane particles) (see FIG. 3 of Blum et al., TIG 5(5):154-158, 1989) in the Bam HI site of pBR322 (Moriarty et al., *Proc. Natl. Acad. Sci. USA* 78:2606-2610, 1981).

Alternatively, cDNA sequences which encode the above-described heterologous sequences may be obtained from cells which express or contain the sequences. Briefly, within one embodiment, mRNA from a cell which expresses the gene of interest is reverse transcribed with reverse transcriptase using oligonucleotide dT or random primers. The single stranded cDNA may then be amplified by PCR (see U.S. Pat. Nos. 4,683,202; 4,683,195 and 4,800,159. See also *PCR Technology: Principles and Applications for DNA Amplification*, Erlich (ed.), Stockton Press, 1989) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double-stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence-specific DNA primers, dATP, dCTP, dGTP and dTTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Sequences which encode the above-described proteins may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (e.g., APB DNA synthesizer model 392 (Foster City, Calif.)).

#### F. EUKARYOTIC LAYERED VECTOR INITIATION SYSTEMS

Due to the size of a full-length genomic alphavirus cDNA clone, in vitro transcription of full-length RNA molecules is rather inefficient. This results in a lowered transfection efficiency, in terms of infectious centers of virus (as measured by plaque formation), relative to the amount of in vitro transcribed RNA transfected. Such inefficiency is also relevant to the in vitro transcription of alphavirus expression vectors. Testing of candidate cDNA clones and other alphavirus cDNA expression vectors for their ability to initiate an infectious cycle or to direct the expression of a heterologous sequence would thus be greatly facilitated if a cDNA clone was transfected into susceptible cells as a DNA molecule, which then directed the synthesis of viral RNA in vivo.

Therefore, within one aspect of the present invention DNA-based vectors (referred to as "Eukaryotic Layered Vector Initiation Systems") are provided which are capable of directing the synthesis of viral RNA in vivo. In particular,

eukaryotic layered vector initiation systems are provided comprising a promoter which is capable of initiating the 5' synthesis of RNA from cDNA, a construct which is capable of autonomous replication in a cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription termination. Briefly, such eukaryotic layered vector initiation systems provide a two-stage or "layered" mechanism which controls expression of heterologous nucleotide sequences. The first layer initiates transcription of the second layer, and comprises a promoter which is capable of initiating the 5' synthesis of RNA from cDNA (e.g., a 5' promoter), a 3' transcription termination site, as well as one or more splice sites and/or a polyadenylation site, if desired. Representative promoters suitable for use within the present invention include both eukaryotic (e.g., pol I, II, or III) and prokaryotic promoters, and inducible or non-inducible (i.e., constitutive) promoters, such as, for example, Murine Leukemia virus promoters (e.g., MoMLV), metallothionein promoters, the glucocorticoid promoter, Drosophila actin 5C distal promoter, SV 40 promoter, heat shock protein 65 promoter, heat shock protein 70 promoter, immunoglobulin promoters, Mouse polyoma virus promoter ("Py"), rous sarcoma virus ("RSV"), BK virus and JC virus promoters, MMTV promoter, alphavirus junction region, CMV promoter, Adenovirus VA1RNA, rRNA promoter, tRNA methionine promoter, CaMV 35S promoter, nopaline synthetase promoter, and the lac promoter. The second layer comprises a vector construct which is capable of expressing one or more heterologous nucleotide sequences and of replication in a cell, either autonomously or in response to one or more factors. Within one embodiment of the invention, the second layer construct may be an alphavirus vector construct as described above.

A wide variety of vector systems may be utilized as the first layer of the eukaryotic layered vector initiation system, including for example, viral vector constructs developed from DNA viruses such as those classified in the Poxviridae, including for example canary pox virus or vaccinia virus (e.g., Fisher-Hoch et al., *PNAS* 86:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci.* 569:86-103, 1989; Flexner et al., *Vaccine* 8:17-21, 1990; U.S. Pat. Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); Papoviridae such as BKV, JCV or SV40 (e.g., Mulligan et al., *Nature* 277:108-114, 1979); Adenoviridae, such as adenovirus (e.g., Berkner, *Biotechniques* 6:616-627, 1988; Rosenfeld et al., *Science* 252:431-434, 1991); Parvoviridae, such as adeno-associated virus (e.g., Samulski et al., *J. Vir.* 63:3822-3828, 1989; Mendelson et al., *Virol.* 166:154-165, 1988; PA 7/222,684); Herpesviridae, such as Herpes Simplex Virus (e.g., Kit, *Adv. Exp. Med. Biol.* 215:219-236, 1989); and Hepadnaviridae (e.g., HBV), as well as certain RNA viruses which replicate through a DNA intermediate, such as the Retroviridae (see, e.g., U.S. Pat. No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805; Retroviridae include leukemia in viruses such as MoMLV and immunodeficiency viruses such as HIV, e.g., Poznansky, *J. Virol.* 65:532-536, 1991).

Similarly, a wide variety of vector systems may be utilized as second layer of the eukaryotic layered vector initiation system, including for example, vector systems derived from viruses of the following families: Picornaviridae (e.g., poliovirus, rhinovirus, coxsackieviruses), Caliciviridae, Togaviridae (e.g. alphavirus, rubella), Flaviviridae (e.g., yellow fever), Coronaviridae (e.g., HCV, TGEV, IBV, MHV, BCV), Bunyaviridae, Arenaviridae, Retroviridae (e.g., RSV, MoMLV, HIV, HTLV), hepatitis delta virus and Astrovirus. In addition, non-mammalian RNA

viruses (as well as components derived therefrom) may also be utilized, including for example, bacterial and bacteriophage replicases, as well as components derived from plant viruses, such as potexviruses (e.g., PVX), carlaviruses (e.g., PVM), tobnaviruses (e.g., TRV, PEBV, PRV), Tobamoviruses (e.g., TMV, ToMV, PPMV), luteoviruses (e.g., PLRV), potyviruses (e.g., TEV, PPV, PVY), tombusviruses (e.g., CyRSV), nepoviruses (e.g., GFLV), bromoviruses (e.g., BMV), and topamoviruses.

The replication competency of the autocatalytic vector construct, contained within the second layer of the eukaryotic vector initiation system, may be measured by a variety of assays known to one of skill in the art including, for example, ribonuclease protection assays which measure increases in both positive-sense and negative-sense RNA over time, in transfected cells, in the presence of an inhibitor of cellular RNA synthesis, such as dactinomycin, and assays which measure the synthesis of a subgenomic RNA or expression of a heterologous reporter gene in transfected cells.

Within particularly preferred embodiments of the invention, eukaryotic layered vector initiation systems are provided that comprise a 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA, followed by a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus nonstructural proteins, a viral junction region which is either active or which has been inactivated such that viral transcription of the subgenomic fragment is prevented, an alphavirus RNA polymerase recognition sequence, and a 3' sequence which controls transcription termination. Within various embodiments, the viral junction region may be modified, such that viral transcription of the subgenomic fragment is merely reduced, rather than inactivated. Within other embodiments, a second viral junction region may be inserted following the first inactivated viral junction region, the second viral junction region being either active or modified such that viral transcription of the subgenomic fragment is reduced.

Following transcription of an alphavirus cDNA vector construct, the resulting alphavirus RNA vector molecule is comprised of a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus nonstructural proteins, a viral junction region, a heterologous nucleotide sequence, an alphavirus RNA polymerase recognition sequence, and a polyadenylate sequence.

Various aspects of the alphavirus cDNA vector constructs have been discussed above, including the 5' sequence which is capable of initiating transcription of an alphavirus, the nucleotide sequence encoding alphavirus nonstructural proteins, the viral junction region, including junction regions which have been inactivated such that viral transcription of the subgenomic fragment is prevented, and the alphavirus RNA polymerase recognition sequence. In addition, modified junction regions and tandem junction regions have also been discussed above.

Within certain aspects of the present invention, methods are provided for delivering a heterologous nucleotide sequence to a warm-blooded animal, comprising the step of administering a eukaryotic layered vector initiation system as described above, to a warm-blooded animal. Eukaryotic layered vector initiation systems may be administered to warm-blooded animals either directly (e.g., intravenously, intramuscularly, intraperitoneally, subcutaneously, orally, rectally, intraocularly, intranasally), or by various physical methods such as lipofection (Felgner et al., *Proc. Natl. Acad.*

*Sci. USA* 84:7413-7417, 1989), direct DNA injection (Acsadi et al., *Nature* 352:815-818, 1991); microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991); liposomes of several types (see, e.g., Wang et al., *PNAS* 84:7851-7855, 1987);  $\text{CaPO}_4$  (Dubensky et al., *PNAS* 81:7529-7533, 1984); DNA ligand (Wu et al., *J. of Biol. Chem.* 264:16985-16987, 1989); administration of nucleic acids alone (WO 90/11092); or administration of DNA linked to killed adenovirus (Curiel et al., *Hum. Gene Ther.* 3:147-154, 1992); via polycation compounds such as polylysine, utilizing receptor specific ligands; as well as with psoralen inactivated viruses such as Sendai or Adenovirus. In addition, the eukaryotic layered vector initiation systems may either be administered directly (i.e., in vivo), or to cells which have been removed (ex vivo), and subsequently returned.

Eukaryotic layered vector initiation systems may be administered to a warm-blooded animal for any of the therapeutic uses described herein, including for example, for the purpose of stimulating a specific immune response; inhibiting the interaction of an agent with a host cell receptor; to express a toxic palliative, including for example, conditional toxic palliatives; to immunologically regulate the immune system; to express markers, and for replacement gene therapy. These and other uses are discussed in more detail below.

In another embodiment of this aspect of the invention, eukaryotic layered vector initiation systems can be utilized to direct the expression of one or more recombinant proteins by eukaryotic cells. As used herein, a "recombinant protein" refers to a protein, polypeptide, enzyme, or fragment thereof. Using this approach, proteins having therapeutic or other commercial application can be more cost-effectively produced. Furthermore, proteins produced in eukaryotic cells may be post-translationally modified (e.g., glycosylated, sulfated, acetylated, etc.), as compared to proteins produced in prokaryotic cells. In addition, such systems may be employed in the in vivo production of various chemical compounds, e.g., fine or specialty chemicals.

Within this embodiment, a eukaryotic layered vector initiation system encoding the desired protein, enzyme, or enzymatic pathway (as may be required for the production of a desired chemical) is transformed, transfected, or otherwise introduced into a suitable eukaryotic cell line. Representative examples of proteins which can be produced using such a system include, but are not limited to, insulin (see U.S. Pat. No. 4,431,740 and BE 885196A), hemoglobin (Lawn et al. *Cell* 21:647-51, 1980), erythropoietin (EPO; see U.S. Pat. No. 4,703,008), megakaryocyte growth and differentiation factor (MGDF), stem cell factor (SCF), G-CSF (Nagata et al. *Nature* 319:415-418, 1986), GM-CSF, M-CSF (see WO 8706954), the flt3 ligand (Lyman, et al. (1993), *Cell*, vol. 75, pp. 1157-1167), EGF, acidic and basic FGF, PDGF, members of the interleukin or interferon families, supra, neurotropic factors (e.g., BDNF; Rosenthal et al. *Endocrinology* 129:1289-1294, 1991, NT-3; see WO 9103569, CNTF; see WO 9104316, NGF; see WO 9310150), coagulation factors (e.g., factors VIII and IX), thrombolytic factors such as t-PA (see EP 292009, AU 8653302 and EP 174835) and streptokinase (see EP 407942), human growth hormone (see JP 94030582 and U.S. Pat. No. 4,745,069) and other animal somatotropins, and integrins and other cell adhesion molecules, such as ICAM-1 and ELAM. Genes encoding such recombinant proteins are among the heterologous nucleic acid sequences of the invention. As those in the art will appreciate, once

characterized, any gene can be readily cloned into a eukaryotic layered vector initiation system according to the invention, followed by introduction into a suitable host cell and expression of the desired gene.

In a preferred embodiment of this and other aspects of the invention, the eukaryotic layered vector initiation system is one derived from an alphavirus vector, such as a Sindbis vector construct, which has been adapted to replicate in one or more cell lines from a particular eukaryotic species, especially a mammalian species, such as humans. For instance, if the gene encoding the recombinant protein to be expressed is of human origin and the protein is intended for human therapeutic use, production in a suitable human cell line may be preferred in order that the protein be post-translationally modified as would be expected to occur in humans. This approach may be useful in further enhancing recombinant protein production. Given the overall plasticity of an alphaviral genome due to the infidelity of the viral replicase, variant strains with an enhanced ability to establish high titer productive infection in selected eukaryotic cells (e.g., human, murine, canine, feline, etc.) can be isolated. Additionally, variant alphaviral strains having an enhanced ability to establish high titer persistent infection in eukaryotic cells may also be isolated using this approach. Alphavirus expression vectors can then be constructed from cDNA clones of these variant strains according to procedures provided herein.

Within another preferred embodiment of this aspect of the invention, the eukaryotic layered vector initiation system comprises a promoter for initial alphaviral vector transcription that is transcriptionally active only in a differentiated cell type. It is well established that alphaviral infection of cells in culture, in particular those derived from hamster (e.g., baby hamster kidney cells) or chicken (e.g., chicken embryo fibroblasts), may result in cytotoxicity. Thus, to produce a stably transformed or transfected host cell line, the eukaryotic layered vector initiation system is preferably introduced into a host cell wherein the promoter which enables the initial vector amplification is a transcriptionally inactive, but inducible, promoter. In a particularly preferred embodiment, such a promoter is differentiation state dependent. In this configuration, activation of the promoter and subsequent activation of the alphavirus DNA vector coincides with induction of cell differentiation. Upon growth to a certain cell number of such a stably transformed or transfected host cell line, the appropriate differentiation stimulus is provided, thereby initiating transcription of the vector construct and amplified expression of the desired gene and encoded polypeptide(s). Many such differentiation state-dependent promoters are known to those in the art, as are cell lines which can be induced to differentiate by application of a specific stimulus. Representative examples include cell lines F9 and P 19, HL60, and Freund erythro-leukemic cell lines and HEL, which are activated by retinoic acid, horse serum, and DMSO, respectively.

#### G. ALPHAVIRUS PACKAGING CELL LINES

Within further embodiments of the invention, alphavirus packaging cell lines are provided. In particular, within one aspect of the present invention, alphavirus packaging cell lines are provided wherein the viral structural proteins, supplied in trans from one or more stably integrated expression vectors, are able to encapsidate transfected, transduced, or intracellularly produced vector RNA transcripts in the cytoplasm and release infectious packaged vector particles through the cell membrane, thus creating an alphavirus vector producing cell line. Alphavirus RNA vector molecules, capable of replicating in the cytoplasm of the

packaging cell, can be produced initially utilizing, for example, an SP6 RNA polymerase system to transcribe in vitro a cDNA vector clone encoding the gene of interest and the alphavirus nonstructural proteins (described previously). Vector RNA transcripts are then transfected into the alphavirus packaging cell line, such that the vector RNA replicates to high levels, and is subsequently packaged by the viral structural proteins, yielding infectious vector particles. Because of the extended length of the alphavirus cDNA molecule, the in vitro transcription process is inefficient. Further, only a fraction of the cells contained in a monolayer are typically transfected by most procedures.

In an effort to optimize vector producing cell line performance and titer, two successive cycles of gene transfer may be performed. In particular, rather than directly transfecting alphavirus RNA vector molecules into the final producing cell line, the vector may first be transfected into a primary alphavirus packaging cell line. The transfected primary packaging cell line releases infectious vector particles into the culture supernatants and these vector-containing supernatants are subsequently used to transduce a fresh monolayer of alphavirus packaging cells. Transduction into the final alphavirus vector producing cells is preferred over transfection because of its higher RNA transfer efficiency into cells and optimized biological placement of the vector in the cell. This leads to higher expression and higher titer of packaged infectious recombinant alphavirus vector.

Within certain embodiments of the invention, alphavirus vector particles may fail to transduce the same packaging cell line because the cell line produces extracellular envelope proteins which block cellular receptors for alphavirus vector particle attachment, a second type of alphavirus vector particle is generated which maintains the ability to transduce the alphavirus packaging cells. This second type of viral particle is produced by a packaging cell line known as a "hopping cell line," which produces transient vector particles as the result of being transfected with in vitro transcribed alphavirus RNA vector transcripts. Briefly, the hopping cell line is engineered to redirect the receptor tropism of the transiently produced vector particles by providing alternative viral envelope proteins which redirect alphavirus vectors to different cellular receptors, in a process termed pseudotyping. Two primary approaches have been devised for alphavirus vector particle pseudotyping. The first approach consists of an alphavirus packaging cell line expressing the vesicular stomatitis virus G protein (VSV-G). The second approach for producing a pseudotyped alphavirus vector particle is to use currently available retroviral packaging cell lines containing retroviral gag/pol and env sequences which would be capable of packaging an alphavirus RNA vector containing a retroviral packaging sequence (e.g., WO 92/05266).

Within other embodiments of the invention, a second approach has also been devised in which a stably integrated DNA expression vector is used to produce the alphavirus vector RNA molecule, which, as in the first approach, maintains the autocatalytic ability to self-replicate. This approach allows for continued vector expression over extended periods of culturing because the integrated DNA vector expression system is maintained through a drug selection marker and the DNA system will constitutively express unaltered RNA vectors which cannot be diluted out by defective RNA copies. In this "alphavirus producer cell line" configuration, the DNA-based alphavirus vector is introduced initially into the packaging cell line by transfection, since size restrictions could prevent packaging of the expression vector into a viral vector particle for

transduction. Also, for this configuration, the SP6 RNA polymerase recognition site of the plasmid, previously used to transcribe vector RNA *in vitro*, is replaced with another appropriate promoter sequence defined by the parent cell line used. In addition, this plasmid sequence also contains a selection marker different from that used to create the packaging cell line.

The expression of alphavirus proteins and/or vector RNA above certain levels may result in cytotoxic effects in packaging cell lines. Therefore, within certain embodiments of the invention, it may be desirable for these elements to be expressed only after the packaging/producer cells have been propagated to a certain critical density. For this purpose, additional packaging or producer cell line modifications are made whereby the structural proteins necessary for packaging are synthesized only after induction by the RNA vector itself or some other stimulus. Also, other modifications allow for the individual expression of these proteins under the control of separate inducible elements, by utilizing expression vectors which unlink the genes encoding these proteins. In addition, expression of the integrated vector molecule itself, in some instances, is controlled by yet another inducible system. This configuration results in a cascade of events following induction, that ultimately leads to the production of packaged vector particles.

#### H. METHODS FOR UTILIZING ALPHAVIRUS VECTORS

##### 1. IMMUNOSTIMULATION

Within other aspects of the present invention, compositions and methods are provided for administering an alphavirus vector construct which is capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous, auto-immune or immune diseases. Representative examples of such diseases include viral infections such as HIV, HBV HTLV I, HTLV II, CMV, EBV and HPV, melanomas, diabetes, graft vs. host disease, Alzheimer's disease and heart disease.

More specifically, within one aspect of the present invention, compositions and methods are provided for stimulating an immune response (either humoral or cell-mediated) to a pathogenic agent, such that the pathogenic agent is either killed or inhibited. Representative examples of pathogenic agents include bacteria, fungi, parasites, viruses and cancer cells.

Within one embodiment of the invention the pathogenic agent is a virus, and methods are provided for stimulating a specific immune response and inhibiting viral spread by using recombinant alphavirus viral particles designed to deliver a vector construct that directs the expression of an antigen or modified form thereof to susceptible target cells capable of either (1) initiating an immune response to the viral antigen or (2) preventing the viral spread by occupying cellular receptors required for viral interactions. Expression of the vector nucleic acid encoded protein may be transient or stable with time. Where an immune response is to be stimulated to a pathogenic antigen, the recombinant alphavirus is preferably designed to express a modified form of the antigen which will stimulate an immune response and which has reduced pathogenicity relative to the native antigen. This immune response is achieved when cells present antigens in the correct manner, i.e., in the context of the MHC class I and/or II molecules along with accessory molecules such as CD3, ICAM-1, ICAM-2, LFA-1, or analogues thereof (e.g., Altmann et al., *Nature* 338:512, 1989). Cells infected with alphavirus vectors are expected to do this efficiently because they closely mimic genuine viral infection and because they: (a) are able to infect non-replicating cells, (b) do not

integrate into the host cell genome, (c) are not associated with any life threatening diseases, and (d) express high levels of heterologous protein. Because of these differences, alphavirus vectors can easily be thought of as safe viral vectors which can be used on healthy individuals for vaccine use.

This aspect of the invention has a further advantage over other systems that might be expected to function in a similar manner, in that the presenter cells are fully viable and healthy, and low levels of viral antigens, relative to heterologous genes, are expressed. This presents a distinct advantage since the antigenic epitopes expressed can be altered by selective cloning of sub-fragments of the gene for the antigen into the recombinant alphavirus, leading to responses against immunogenic epitopes which may otherwise be overshadowed by immunodominant epitopes. Such an approach may be extended to the expression of a peptide having multiple epitopes, one or more of the epitopes being derived from different proteins. Further, this aspect of the invention allows efficient stimulation of cytotoxic T lymphocytes (CTL) directed against antigenic epitopes, and peptide fragments of antigens encoded by sub-fragments of genes, through intracellular synthesis and association of these peptide fragments with MHC Class I molecules. This approach may be utilized to map major immunodominant epitopes for CTL induction.

An immune response may also be achieved by transferring to an appropriate immune cell (such as a T lymphocyte) the gene for the specific T cell receptor which recognizes the antigen of interest (in the context of an appropriate MHC molecule if necessary), for an immunoglobulin which recognizes the antigen of interest, or for a hybrid of the two which provides a CTL response in the absence of the MHC context. Thus, the recombinant alphavirus infected cells may be used as an immunostimulant, immunomodulator, or vaccine.

In another embodiment of the invention, methods are provided for producing inhibitor palliatives wherein alphavirus vectors deliver and express defective interfering viral structural proteins, which inhibit viral assembly. Such vectors may encode defective gag, pol, env or other viral particle proteins or peptides and these would inhibit in a dominant fashion the assembly of viral particles. This occurs because the interaction of normal subunits of the viral particle is disturbed by interaction with the defective subunits.

In another embodiment of the invention, methods are provided for the expression of inhibiting peptides or proteins specific for viral protease. Briefly, viral protease cleaves the viral gag and gag/pol proteins into a number of smaller peptides. Failure of this cleavage in all cases leads to complete inhibition of production of infectious retroviral particles. As an example, the HIV protease is known to be an aspartyl protease and these are known to be inhibited by peptides made from amino acids from protein or analogues. Vectors to inhibit HIV will express one or multiple fused copies of such peptide inhibitors.

Another embodiment involves the delivery of suppressor genes which, when deleted, mutated, or not expressed in a cell type, lead to tumorigenesis in that cell type. Reintroduction of the deleted gene by means of a viral vector leads to regression of the tumor phenotype in these cells. Examples of such cancers are retinoblastoma and Wilms Tumor. Since malignancy can be considered to be an inhibition of cellular terminal differentiation compared with cell growth, the alphavirus vector delivery and expression of gene products which lead to differentiation of a tumor should also, in general, lead to regression.

In yet another embodiment, the alphavirus vector provides a therapeutic effect by transcribing a ribozyme (an RNA enzyme) (Haseloff and Gerlach, *Nature* 334:585, 1989) which will cleave and hence inactivate RNA molecules corresponding to a pathogenic function. Since ribozymes function by recognizing a specific sequence in the target RNA and this sequence is normally 12 to 17 bp, this allows specific recognition of a particular RNA species such as a RNA or a retroviral genome. Additional specificity may be achieved in some cases by making this a conditional toxic palliative (see below).

One way of increasing the effectiveness of inhibitory palliatives is to express viral inhibitory genes in conjunction with the expression of genes which increase the probability of infection of the resistant cell by the virus in question. The result is a nonproductive "dead-end" event which would compete for productive infection events. In the specific case of HIV, vectors may be delivered which inhibit HIV replication (by expressing anti-sense tat, etc., as described above) and also overexpress proteins required for infection, such as CD4. In this way, a relatively small number of vector-infected HIV-resistant cells act as a "sink" or "magnet" for multiple nonproductive fusion events with free virus or virally infected cells.

## 2. BLOCKING AGENTS

Many infectious diseases, cancers, autoimmune diseases, and other diseases involve the interaction of viral particles with cells, cells with cells, or cells with factors. In viral infections, viruses commonly enter cells via receptors on the surface of susceptible cells. In cancers, cells may respond inappropriately or not at all to signals from other cells or factors. In autoimmune disease, there is inappropriate recognition of "self" markers. Within the present invention, such interactions may be blocked by producing, in vivo, an analogue to either of the partners in an interaction.

This blocking action may occur intracellularly, on the cell membrane, or extracellularly. The blocking action of a viral or, in particular, an alphavirus vector carrying a gene for a blocking agent, can be mediated either from inside a susceptible cell or by secreting a version of the blocking protein to locally block the pathogenic interaction.

In the case of HIV, the two agents of interaction are the gp 120/gp 41 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker would be a vector construct expressing either an HIV env analogue that blocks HIV entry without causing pathogenic effects, or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp 41 is secreted or produced only intracellularly so as to protect only the vector-containing cell. It may be advantageous to add human immunoglobulin heavy chains or other components to CD4 in order to enhance stability or complement lysis. Delivery of an alphavirus vector encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule. Efficacy of treatment can be assayed by measuring the usual indicators of disease progression, including antibody level, viral antigen production, infectious HIV levels, or levels of nonspecific infections.

## 3. EXPRESSION OF PALLIATIVES

Techniques similar to those described above can be used to produce recombinant alphavirus vector constructs which direct the expression of an agent (or "palliative") which is capable of inhibiting a function of a pathogenic agent or gene. Within the present invention, "capable of inhibiting a function" means that the palliative either directly inhibits the function or indirectly does so, for example, by converting an

agent present in the cells from one which would not normally inhibit a function of the pathogenic agent to one which does. Examples of such functions for viral diseases include adsorption, replication, gene expression, assembly, and exit of the virus from infected cells. Examples of such functions for a cancerous cell or cancer-promoting growth factor include viability, cell replication, altered susceptibility to external signals (e.g., contact inhibition), and lack of production or production of mutated forms of anti-oncogene proteins.

### (a) Inhibitor Palliatives

In one aspect of the present invention, the alphavirus vector construct directs the expression of a gene which can interfere with a function of a pathogenic agent, for instance in viral or malignant diseases. Such expression may either be essentially continuous or in response to the presence in the cell of another agent associated either with the pathogenic condition or with a specific cell type (an "identifying agent"). In addition, vector delivery may be controlled by targeting vector entry specifically to the desired cell type (for instance, a virally infected or malignant cell) as discussed above.

One method of administration is leukapheresis, in which about 20% of an individual's PBLs are removed at any one time and manipulated in vitro. Thus, approximately  $2 \times 10^9$  cells may be treated and replaced. Repeat treatments may also be performed. Alternatively, bone marrow may be treated and allowed to amplify the effect as described above. In addition, packaging cell lines producing a vector may be directly injected into a subject, allowing continuous production of recombinant virions.

In one embodiment, alphavirus vectors which express RNA complementary to key pathogenic gene transcripts (for example, a viral gene product or an activated cellular oncogene) can be used to inhibit translation of that transcript into protein, such as the inhibition of translation of the HIV tat protein. Since expression of this protein is essential for viral replication, cells containing the vector would be resistant to HIV replication.

In a second embodiment, where the pathogenic agent is a single-stranded virus having a packaging signal, RNA complementary to the viral packaging signal (e.g., an HIV packaging signal when the palliative is directed against HIV) is expressed, so that the association of these molecules with the viral packaging signal will, in the case of retroviruses, inhibit stem loop formation or tRNA primer binding required for proper encapsidation or replication of the alphavirus RNA genome.

In a third embodiment, an alphavirus vector may be introduced which expresses a palliative capable of selectively inhibiting the expression of a pathogenic gene, or a palliative capable of inhibiting the activity of a protein produced by the pathogenic agent. In the case of HIV, one example is a mutant tat protein which lacks the ability to transactivate expression from the HIV LTR and interferes (in a transdominant manner) with the normal functioning of tat protein. Such a mutant has been identified for HTLV II tat protein ("XII Leu<sup>5</sup>" mutant; see Wachsmann et al., *Science* 235:674, 1987). A mutant transrepressor tat should inhibit replication much as has been shown for an analogous mutant repressor in HSV-1 (Friedmann et al., *Nature* 335:452, 1988).

Such a transcriptional repressor protein may be selected for in tissue culture using any viral-specific transcriptional promoter whose expression is stimulated by a virus-specific transactivating protein (as described above). In the specific case of HIV, a cell line expressing HIV tat protein and the



HSVTK gene driven by the HIV promoter will die in the presence of ACV. However, if a series of mutated tat genes are introduced to the system, a mutant with the appropriate properties (i.e., represses transcription from the HIV promoter in the presence of wild-type tat) will grow and be selected. The mutant gene can then be reisolated from these cells. A cell line containing multiple copies of the conditionally lethal vector/tat system may be used to assure that surviving cell clones are not caused by endogenous mutations in these genes. A battery of randomly mutagenized tat genes are then introduced into these cells using a "rescuable" alphavirus vector (i.e., one that expresses the mutant tat protein and contains a bacterial origin of replication and drug resistance marker for growth and selection in bacteria). This allows a large number of random mutations to be evaluated and permits facile subsequent molecular cloning of the desired mutant cell line. This procedure may be used to identify and utilize mutations in a variety of viral transcriptional activator/viral promoter systems for potential antiviral therapies.

#### 4. CONDITIONAL TOXIC PALLIATIVES

Another approach for inhibiting a pathogenic agent is to express a palliative which is toxic for the cell expressing the pathogenic condition. In this case, expression of the palliative from the vector should be limited by the presence of an entity associated with the pathogenic agent, such as a specific viral RNA sequence identifying the pathogenic state, in order to avoid destruction of nonpathogenic cells.

In one embodiment of this method, a recombinant alphavirus vector carries a vector construct containing a toxic gene (as discussed above) expressed from a cell-specific responsive vector. In this manner, rapidly replicating cells, which contain the RNA sequences capable of activating the cell-specific responsive vectors, are preferentially destroyed by the cytotoxic agent produced by the alphavirus vector construct.

In a similar manner to the preceding embodiment, the alphavirus vector construct can carry a gene for phosphorylation, phosphoribosylation, ribosylation, or other metabolism of a purine- or pyrimidine-based drug. This gene may have no equivalent in mammalian cells and might come from organisms such as a virus, bacterium, fungus, or protozoan. An example of this would be the *E. coli* guanine phosphoribosyl transferase gene product, which is lethal in the presence of thioxanthine (see Besnard et al., *Mol. Cell Biol.* 7:4139-4141, 1987). Conditionally lethal gene products of this type (also referred to as "pro-drugs" above) have application to many presently known purine- or pyrimidine-based anticancer drugs, which often require intracellular ribosylation or phosphorylation in order to become effective cytotoxic agents. The conditionally lethal gene product could also metabolize a nontoxic drug which is not a purine or pyrimidine analogue to a cytotoxic form (see Searle et al., *Brit. J. Cancer* 53:377-384, 1986).

Mammalian viruses in general tend to have "immediate early" genes which are necessary for subsequent transcriptional activation from other viral promoter elements. RNA sequences of this nature are excellent candidates for activating alphavirus vectors intracellular signals (or "identifying agents") of viral infection. Thus, conditionally lethal genes expressed from alphavirus cell-specific vectors responsive to these viral "immediate early" gene products could specifically kill cells infected with any particular virus. Additionally, since the human and interferon promoter elements are transcriptionally activated in response to infection by a wide variety of nonrelated viruses, the introduction of vectors expressing a conditionally lethal gene product like

HSVTK, for example, in response to interferon production could result in the destruction of cells infected with a variety of different viruses.

In another aspect of the present invention, the recombinant alphavirus viral vector carries a vector construct that directs the expression of a gene product capable of activating an otherwise inactive precursor into an active inhibitor of the pathogenic agent. For example, the HSVTK gene product may be used to more effectively metabolize potentially antiviral nucleoside analogues such as AZT or ddC. The HSVTK gene may be expressed under the control of a cell-specific responsive vector and introduced into these cell types. AZT (and other nucleoside antivirals) must be metabolized by cellular mechanisms to the nucleotide triphosphate form in order to specifically inhibit retroviral reverse transcriptase, and thus, HIV replication (Furman et al., *Proc. Natl. Acad. Sci. USA* 83:8333-8337, 1986). Constitutive expression of HSVTK (a nucleoside and nucleoside kinase with very broad substrate specificity) results in more effective metabolism of these drugs to their biologically active nucleotide triphosphate form. AZT or ddC therapy will thereby be more effective, allowing lower doses, less generalized toxicity, and higher potency against productive infection. Additional nucleoside analogues whose nucleotide triphosphate forms show selectivity for retroviral reverse transcriptase but, as a result of the substrate specificity of cellular nucleoside and nucleotide kinases are not phosphorylated, will be made more efficacious.

Administration of these alphavirus vectors to human T cell and macrophage/monocyte cell lines can increase their resistance to HIV in the presence of AZT and ddC compared to the same cells without retroviral vector treatment. Treatment with AZT would be at lower than normal levels to avoid toxic side effects but still efficiently inhibit the spread of HIV. The course of treatment would be as described for the blocker.

In one embodiment, the recombinant alphavirus vector carries a gene specifying a product which is not in itself toxic but, when processed or modified by a protein such as a protease specific to a viral or other pathogen, is converted into a toxic form. For example, the recombinant alphavirus could carry a gene encoding a proprotein for ricin A chain, which becomes toxic upon processing by the HIV protease. More specifically, a synthetic inactive proprotein form of the toxin ricin or diphtheria A chains could be cleaved to the active form by arranging for the HIV virally encoded protease to recognize and cleave off an appropriate "pro" element.

In another embodiment, the alphavirus construct may express a "reporting product" on the surface of the target cells in response to the presence of an identifying agent in the cells (such as expression of a viral gene). This surface protein can be recognized by a cytotoxic agent, such as antibodies for the reporting protein, or by cytotoxic T cells. In a similar manner, such a system can be used as a detection system (see below) to simply identify those cells having a particular gene which expresses an identifying protein.

Similarly, in another embodiment, a surface protein could be expressed which would itself be therapeutically beneficial. In the particular case of HIV, expression of the human CD4 protein specifically in HIV-infected cells may be beneficial in two ways:

1. Binding of CD4 to HIV env intracellularly could inhibit the formation of viable viral particles, much as soluble CD4 has been shown to do for free virus, but without the problem of systematic clearance and possible immunogenicity, since the protein will remain mem-



brane bound and is structurally identical to endogenous CD4 (to which the patient should be immunologically tolerant).

2. Since the CD4/HIV env complex has been implicated as a cause of cell death, additional expression of CD4 (in the presence of excess HIV-env present in HIV-infected cells) leads to more rapid cell death and thus inhibits viral dissemination. This may be particularly applicable to monocytes and macrophages, which act as a reservoir for virus production as a result of their relative refractility to HIV-induced cytotoxicity (which, in turn, is apparently due to the relative lack of CD4 on their cell surfaces).

In another embodiment, the alphavirus vector codes for a ribozyme which will cleave and inactivate RNA molecules essential for viability of the vector infected cell. By making ribozyme production dependent on a specific RNA sequence corresponding to the pathogenic state, such as HIV tat, toxicity is specific to the pathogenic state.

#### 5. EXPRESSION OF MARKERS

The above-described technique of expressing a palliative in a cell in response to a specific RNA sequence can also be modified to enable detection of a particular gene in a cell which expresses an identifying protein (for example, a gene carried by a particular virus), and hence enable detection of cells carrying that virus. In addition, this technique enables the detection of viruses (such as HIV) in a clinical sample of cells carrying an identifying protein associated with the virus.

This modification can be accomplished by providing a genome coding for a product, the presence of which can be readily identified (the "marker product"), in an alphavirus vector which responds to the presence of the identifying protein in the infected cells. For example, HIV, when it infects suitable cells, makes tat and rev. The indicator cells can thus be provided with a genome (such as by infection with an appropriate recombinant alphavirus) which codes for a marker gene, such as the alkaline phosphatase gene,  $\beta$ -galactosidase gene, or the luciferase gene which is expressed by the recombinant alphavirus upon activation by the tat and/or rev RNA transcript. In the case of  $\beta$ -galactosidase or alkaline phosphatase, exposing the cells to substrate analogues results in a color or fluorescence change if the sample is positive for HIV. In the case of luciferase, exposing the sample to luciferin will result in luminescence if the sample is positive for HIV. For intracellular enzymes such as  $\beta$ -galactosidase, the viral titre can be measured directly by counting colored or fluorescent cells, or by making cell extracts and performing a suitable assay. For the membrane bound form of alkaline phosphatase, virus titre can also be measured by performing enzyme assays on the cell surface using a fluorescent substrate. For secreted enzymes, such as an engineered form of alkaline phosphatase, small samples of culture supernatant are assayed for activity, allowing continuous monitoring of a single culture over time. Thus, different forms of this marker system can be used for different purposes. These include counting active virus, or sensitively and simply measuring viral spread in a culture and the inhibition of this spread by various drugs.

Further specificity can be incorporated into the preceding system by testing for the presence of the virus either with or without neutralizing antibodies to that virus. For example, in one portion of the clinical sample being tested, neutralizing antibodies to HIV may be present; whereas in another portion there would be no neutralizing antibodies. If the tests were negative in the system where there were antibodies and

positive where there were no antibodies, this would assist in confirming the presence of HIV.

Within an analogous system for an in vitro assay, the presence of a particular gene, such as a viral gene, may be determined in a cell sample. In this case, the cells of the sample are infected with a suitable alphavirus vector which carries the reporter gene which is only expressed in the presence of the appropriate viral RNA transcript. The reporter gene, after entering the sample cells, will express its reporting product (such as  $\beta$ -galactosidase or luciferase) only if the host cell expresses the appropriate viral proteins.

These assays are more rapid and sensitive, since the reporter gene can express a greater amount of reporting product than identifying agent present, which results in an amplification effect.

#### 6. IMMUNE DOWN-REGULATION

As briefly described above, the present invention also provides recombinant alphavirus which carry a vector construct capable of suppressing one or more elements of the immune system in target cells infected with the alphavirus.

Briefly, specific down-regulation of inappropriate or unwanted immune responses, such as in chronic hepatitis or in transplants of heterologous tissue such as bone marrow, can be engineered using immune-suppressive viral gene products which suppress surface expression of transplantation (MHC) antigen. Group C adenoviruses Ad2 and Ad5 possess a 19 kd glycoprotein (gp 19) encoded in the E3 region of the virus. This gp 19 molecule binds to class I MHC molecules in the endoplasmic reticulum of cells, and prevents terminal glycosylation and translocation of class I MHC to the cell surface. For example, prior to bone marrow transplantation, donor bone marrow cells may be infected with gp 19-encoding vector constructs which, upon expression of the gp 19, inhibit the surface expression of MHC class I transplantation antigens. These donor cells may be transplanted with low risk of graft rejection and may require a minimal immunosuppressive regimen for the transplant patient. This may allow an acceptable donor-recipient chimeric state to exist with fewer complications. Similar treatments may be used to treat the range of so-called autoimmune diseases, including lupus erythematosus, multiple sclerosis, rheumatoid arthritis or chronic hepatitis B infection.

An alternative method involves the use of anti-sense message, ribozyme, or other specific gene expression inhibitor specific for T cell clones which are autoreactive in nature. These block the expression of the T cell receptor of particular unwanted clones responsible for an autoimmune response. The anti-sense, ribozyme, or other gene may be introduced using the viral vector delivery system.

#### 7. REPLACEMENT OR AUGMENTATION GENE THERAPY

One further aspect of the present invention relates to transforming cells of an animal with recombinant alphavirus vectors which serve as gene transfer vehicles to supply genetic sequences capable of expressing a therapeutic protein. Within one embodiment of the present invention, the viral vector construct is designed to express a therapeutic protein capable of preventing, inhibiting, stabilizing or reversing an inherited or noninherited genetic defect in metabolism, immune regulation, hormonal regulation, enzymatic or membrane associated structural function. This embodiment also describes the viral vector capable of transducing individual cells, whereby the therapeutic protein is able to be expressed systemically or locally from a specific cell or tissue, whereby the therapeutic protein is capable of (a) the replacement of an absent or defective cellular protein

or enzyme, or (b) supplement production of a defective or low expressed cellular protein or enzyme. Such diseases may include cystic fibrosis, Parkinson's disease, hypercholesterolemia, adenosine deaminase deficiency,  $\beta$ -globin disorders, Hemophilia A & B, Gaucher's disease, diabetes and leukemia.

As an example of the present invention, a recombinant alphavirus viral vector can be used to treat Gaucher disease. Briefly, Gaucher disease is a genetic disorder that is characterized by the deficiency of the enzyme glucocerebrosidase. This type of therapy is an example of a single gene replacement therapy by providing a functional cellular enzyme. This enzyme deficiency leads to the accumulation of glucocerebroside in the lysosomes of all cells in the body. However, the disease phenotype is manifested only in the macrophages, except in the very rare neuronopathic forms of the disease. The disease usually leads to enlargement of the liver and spleen and lesions in the bones. (For a review, see *Science* 256:794, 1992, and *The Metabolic Basis of Inherited Disease*, 6th ed., Scriver et al., vol. 2, p. 1677).

#### 8. LYMPHOKINES AND LYMPHOKINE RECEPTORS

As noted above, the present invention provides alphavirus particles which can, among other functions, direct the expression of one or more cytokines or cytokine receptors.

Briefly, in addition to their role as cancer therapeutics, cytokines can have negative effects resulting in certain pathological conditions. For example, most resting T-cells, B cells, large granular lymphocytes and monocytes do not express IL-2R (receptor). In contrast to the lack of IL-2R expression on normal resting cells, IL-2R is expressed by abnormal cells in patients with certain leukemias (ATL, Hairy-cell, Hodgkins, acute and chronic granulocytic), autoimmune diseases, and is associated with allograft rejection. Interestingly, in most of these patients the serum concentration of a soluble form of IL-2R is elevated. Therefore, with certain embodiments of the invention therapy may be effected by increasing the serum concentration of the soluble form of the cytokine receptor. For example, in the case of IL-2R, an alphavirus vector can be engineered to produce both soluble IL-2R and IL-2R, creating a high affinity soluble receptor. In this configuration, serum IL-2 levels would decrease, inhibiting the paracrine loop.

This same strategy may also be effective against autoimmune diseases. In particular, because some autoimmune diseases (e.g., Rheumatoid arthritis, SLE) are also associated with abnormal expression of IL-2, blocking the action of IL-2 by increasing serum level of receptor may also be utilized in order to treat such autoimmune diseases.

In other cases inhibiting the levels of IL-1 may be beneficial. Briefly, IL-1 consists of two polypeptides, IL-1 and IL-1 $\beta$ , each of which has pleiotropic effects. IL-1 is primarily synthesized by mononuclear phagocytes, in response to stimulation by microbial products or inflammation. There is a naturally occurring antagonist of the IL-1R, referred to as the IL-1 Receptor antagonist ("IL-1Ra"). This IL-1R antagonist has the same molecular size as mature IL-1 and is structurally related to it. However, binding of IL-1Ra to the IL-1R does not initiate any receptor signaling. Thus, this molecule has a different mechanism of action than a soluble receptor, which complexes with the cytokine and thus prevents interaction with the receptor. IL-1 does not seem to play an important role in normal homeostasis. In animals, antibodies to IL-1 receptors reduce inflammation and anorexia due to endotoxins and other inflammation inducing agents.

In the case of septic shock, IL-1 induces secondary compounds which are potent vasodilators. In animals, exog-

enously supplied IL-1 decreases mean arterial pressure and induces leukopenia. Neutralizing antibody to IL-1 reduced endotoxin-induced fever in animals. In a study of patients with septic shock who were treated with a constant infusion of IL-1R for three days, the 28 day mortality was 16% compared to 44% in patients who received placebo infusions.

In the case of autoimmune disease, reducing the activity of IL-1 reduces inflammation. Similarly, blocking the activity of IL-1 with recombinant receptors can result in increased allograft survival in animals, again presumably by decreasing inflammation.

These diseases provide further examples where alphavirus vectors may be engineered to produce a soluble receptor or more specifically the IL-1Ra molecule. For example, in patients undergoing septic shock, a single injection of IL-1Ra producing vector particles could replace the current approach requiring a constant infusion of recombinant IL-1R.

Cytokine responses, or more specifically, incorrect cytokine responses may also be involved in the failure to control or resolve infectious diseases. Perhaps the best studied example is non-healing forms of leishmaniasis in mice and humans which have strong, but counterproductive  $T_H$ -2-dominated responses. Similarly, lepromatous leprosy is associated with a dominant, but inappropriate  $T_H$ -2 response. In these conditions, alphavirus-based gene therapy may be useful for increasing circulating levels of IFN gamma, as opposed to the site-directed approach proposed for solid tumor therapy. IFN gamma is produced by  $T_H$ -1 T-cells, and functions as a negative regulator of  $T_H$ -2 subtype proliferation. IFN gamma also antagonizes many of the IL-4 mediated effects on B-cells, including isotype switching to IgE.

IgE, mast cells and eosinophils are involved in mediating allergic reaction. IL-4 acts on differentiating T-cells to stimulate  $T_H$ -2 development, while inhibiting  $T_H$ -1 responses. Thus, alphavirus-based gene therapy may also be accomplished in conjunction with traditional allergy therapeutics. One possibility is to deliver alphavirus-IL4R with small amounts of the offending allergen (i.e., traditional allergy shots). Soluble IL-4R would prevent the activity of IL-4, and thus prevent the induction of a strong  $T_H$ -2 response.

#### 9. SUICIDE VECTOR

One further aspect of the present invention relates to the expression of alphavirus suicide vectors to limit the spread of wild-type alphavirus in the packaging/producer cell lines. Briefly, within one embodiment the alphavirus suicide vector would be comprised of an antisense or ribozyme sequence, specific for the wild-type alphavirus sequence generated from an RNA recombination event between the 3' sequences of the junction region of the vector, and the 5' alphavirus structural sequences of the packaging cell line expression vector. The antisense or ribozyme molecule would only be thermostable in the presence of the specific recombination sequence and would not have any other effect in the alphavirus packaging/producer cell line. Alternatively, a toxic molecule (such as those disclosed below), may also be expressed in the context of a vector that would only express in the presence of wild-type alphavirus.

#### 10. ALPHAVIRUS VECTORS TO PREVENT THE SPREAD OF METASTATIC TUMORS

One further aspect of the present invention relates to the use of alphavirus vectors for inhibiting or reducing the invasiveness of malignant neoplasms. Briefly, the extent of malignancy typically relates to vascularization of the tumor. One cause for tumor vascularization is the production of

soluble tumor angiogenesis factors (TAF) (Pawelitz et al., *Crit. Rev. Oncol. Hematol.* 9:197, 1989) expressed by some tumors. Within one aspect of the present invention, tumor vascularization may be slowed by using alphavirus vectors to express antisense or ribozyme RNA molecules specific for TAF. Alternatively, anti-angiogenesis factors (Moses et al., *Science* 248:1408, 1990; Shapiro et al., *PNAS* 84:2238, 1987) may be expressed either alone or in combination with the above-described ribozymes or antisense sequences in order to slow or inhibit tumor vascularization. Alternatively, alphavirus vectors can also be used to express an antibody specific for the TAF receptors on surrounding tissues.

#### 11. ADMINISTRATION OF ALPHAVIRUS PARTICLES

Within other aspects of the present invention, methods are provided for administering recombinant alphavirus vectors or particles. Briefly, the final mode of viral vector administration usually relies on the specific therapeutic application, the best mode of increasing vector potency, and the most convenient route of administration. Generally, this embodiment includes recombinant alphavirus vectors which can be designed to be delivered by, for example, (1) direct injection into the blood stream; (2) direct injection into a specific tissue or tumor; (3) oral administration; (4) nasal inhalation; (5) direct application to mucosal tissues; or (6) ex vivo administration of transduced autologous cells into the animal. Thus the therapeutic alphavirus vector can be administered in such a fashion such that the vector can (a) transduce a normal healthy cell and transform the cell into a producer of a therapeutic protein or agent which is secreted systemically or locally, (b) transform an abnormal or defective cell, transforming the cell into a normal functioning phenotype, (c) transform an abnormal cell so that it is destroyed, and/or (d) transduce cells to manipulate the immune response.

#### I. MODULATION OF TRANSCRIPTION FACTOR ACTIVITY

In yet another embodiment, alphavirus vectors may be utilized in order to regulate the growth control activity of transcription factors in the infected cell. Briefly, transcription factors directly influence the pattern of gene expression through sequence-specific trans-activation or repression (Karin, *New Biologist* 21:126-131, 1990). Thus, it is not surprising that mutated transcription factors represent a family of oncogenes. Alphavirus gene transfer therapy can be used, for example, to return control to tumor cells whose unregulated growth is activated by oncogenic transcription factors, and proteins which promote or inhibit the binding cooperatively in the formation of homo- and heterodimer trans-activating or repressing transcription factor complexes.

One method for reversing cell proliferation would be to inhibit the trans-activating potential of the c-myc/Max heterodimer transcription factor complex. Briefly, the nuclear oncogene c-myc is expressed by proliferating cells and can be activated by several distinct mechanisms, including retroviral insertion, amplification, and chromosomal translocation. The Max protein is expressed in quiescent cells and, independently of c-myc, either alone or in conjunction with an unidentified factor, functions to repress expression of the same genes activated by the myc/Max heterodimer (Cole, *Cell* 65:715-716, 1991).

Inhibition of c-myc or c-myc/Max proliferation of tumor cells may be accomplished by the overexpression of Max in target cells controlled by alphavirus vectors. The Max protein is only 160 amino acids (corresponding to 480 nucleotide RNA length) and is easily incorporated into an alphavirus vector either independently, or in combination

with other genes and/or antisense/ribozyme moieties targeted to factors which release growth control of the cell.

Modulation of homo/hetero-complex association is another approach to control transcription factor activated gene expression. For example, transport from the cytoplasm to the nucleus of the trans-activating transcription factor NF-B is prevented while in a heterodimer complex with the inhibitor protein IB. Upon induction by a variety of agents, including certain cytokines, IB becomes phosphorylated and NF-B is released and transported to the nucleus, where it can exert its sequence-specific trans-activating function (Baeuerle and Baltimore, *Science* 242:540-546, 1988). The dissociation of the NF-B/IB complex can be prevented by masking with an antibody the phosphorylation site of IB. This approach would effectively inhibit the trans-activation activity of the NF-IB transcription factor by preventing its transport to the nucleus. Expression of the IB phosphorylation site specific antibody or protein in target cells may be accomplished with an alphavirus gene transfer vector. An approach similar to the one described here could be used to prevent the formation of the trans-activating transcription heterodimer factor AP-1 (Turner and Tijan, *Science* 243:1689-1694, 1989), by inhibiting the association between the jun and fos proteins.

#### J. PHARMACEUTICAL COMPOSITIONS

As noted above, the present invention also provides pharmaceutical compositions comprising a recombinant Sindbis particle or virus, or Sindbis vector construct, in combination with a pharmaceutically acceptable carrier, diluent, or recipient.

Briefly, infectious recombinant virus (also referred to above as particles) may be preserved either in crude or purified forms. In order to produce virus in a crude form, virus-producing cells may first be cultivated in a bioreactor, wherein viral particles are released from the cells into the culture media. Virus may then be preserved in crude form by first adding a sufficient amount of a formulation buffer to the culture media containing the recombinant virus to form an aqueous suspension. Within certain preferred embodiments, the formulation buffer is an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. The aqueous solution may also contain one or more amino acids.

The recombinant virus can also be preserved in a purified form. More specifically, prior to the addition of the formulation buffer, the crude recombinant virus described above may be clarified by passing it through a filter and then concentrated, such as by a cross flow concentrating system (Filtron Technology Corp., Northborough, Mass.). Within one embodiment, DNase is added to the concentrate to digest exogenous DNA. The digest is then diafiltrated in order to remove excess media components and to establish the recombinant virus in a more desirable buffered solution. The diafiltrate is then passed over a Sephadex S-500 gel column and a purified recombinant virus is eluted. A sufficient amount of formulation buffer is then added to this eluate in order to reach a desired final concentration of the constituents and to minimally dilute the recombinant virus. The aqueous suspension may then be stored, preferably at -70° C., or immediately dried. As above, the formulation buffer may be an aqueous solution that contains a saccharide, a high molecular weight structural additive, and a buffering component in water. The aqueous solution may also contain one or more amino acids.

Crude recombinant virus may also be purified by ion exchange column chromatography. Briefly, crude recombinant virus may be clarified by first passing it through a filter,

followed by loading the filtrate onto a column containing a highly sulfonated cellulose matrix. The recombinant virus may then be eluted from the column in purified form by using a high salt buffer, and the high salt buffer exchanged for a more desirable buffer by passing the eluate over a molecular exclusion column. A sufficient amount of formulation buffer is then added, as discussed above, to the purified recombinant virus and the aqueous suspension is either dried immediately or stored, preferably at  $-70^{\circ}\text{C}$ .

The aqueous suspension in crude or purified form can be dried by lyophilization or evaporation at ambient temperature. Briefly, lyophilization involves the steps of cooling the aqueous suspension below the gas transition temperature or below the eutectic point temperature of the aqueous suspension, and removing water from the cooled suspension by sublimation to form a lyophilized virus. Within one embodiment, aliquots of the formulated recombinant virus are placed into an Edwards Refrigerated Chamber (3 shelf RC3S unit) attached to a freeze dryer (Supermodulyo 12K). A multistep freeze drying procedure as described by Phillips et al. (*Cryobiology* 18:414, 1981) is used to lyophilize the formulated recombinant virus, preferably from a temperature of  $-40^{\circ}\text{C}$  to  $-45^{\circ}\text{C}$ . The resulting composition contains less than 10% water by weight of the lyophilized virus. Once lyophilized, the recombinant virus is stable and may be stored at  $-20^{\circ}\text{C}$  to  $25^{\circ}\text{C}$ , as discussed in more detail below.

Within the evaporative method, water is removed from the aqueous suspension at ambient temperature by evaporation. Within one embodiment, water is removed through spray-drying (EP 520,748). Within the spray-drying process, the aqueous suspension is delivered into a flow of preheated gas, usually air, whereupon water rapidly evaporates from droplets of the suspension. Spray-drying apparatus are available from a number of manufacturers (e.g., Drytec, Ltd., Tonbridge, England; Lab-Plant, Ltd., Huddersfield, England). Once dehydrated, the recombinant virus is stable and may be stored at  $-20^{\circ}\text{C}$  to  $25^{\circ}\text{C}$ . Within the methods described herein, the resulting moisture content of the dried or lyophilized virus may be determined through use of a Karl-Fischer apparatus (EM Science Aquastar V1B volumetric titrator, Cherry Hill, N.J.), or through a gravimetric method.

The aqueous solutions used for formulation, as previously described, are preferably composed of a saccharide, high molecular weight structural additive, a buffering component, and water. The solution may also include one or more amino acids. The combination of these components act to preserve the activity of the recombinant virus upon freezing and lyophilization or drying through evaporation. Although a preferred saccharide is lactose, other saccharides may be used, such as sucrose, mannitol, glucose, trehalose, inositol, fructose, maltose or galactose. In addition, combinations of saccharides can be used, for example, lactose and mannitol, or sucrose and mannitol. A particularly preferred concentration of lactose is 3%–4% by weight. Preferably, the concentration of the saccharide ranges from 1% to 12% by weight.

The high molecular weight structural additive aids in preventing viral aggregation during freezing and provides structural support in the lyophilized or dried state. Within the context of the present invention, structural additives are considered to be of "high molecular weight" if they are greater than 5000 m.w. A preferred high molecular weight structural additive is human serum albumin. However, other substances may also be used, such as hydroxyethyl-cellulose, hydroxymethyl-cellulose, dextran, cellulose,

gelatin, or povidone. A particularly preferred concentration of human serum albumin is 0.1% by weight. Preferably, the concentration of the high molecular weight structural additive ranges from 0.1% to 10% by weight.

The amino acids, if present, function to further preserve viral infectivity upon cooling and thawing of the aqueous suspension. In addition, amino acids function to further preserve viral infectivity during sublimation of the cooled aqueous suspension and while in the lyophilized state. A preferred amino acid is arginine, but other amino acids such as lysine, ornithine, serine, glycine, glutamine, asparagine, glutamic acid or aspartic acid can also be used. A particularly preferred arginine concentration is 0.1% by weight. Preferably, the amino acid concentration ranges from 0.1% to 10% by weight.

The buffering component acts to buffer the solution by maintaining a relatively constant pH. A variety of buffers may be used, depending on the pH range desired, preferably between 7.0 and 7.8. Suitable buffers include phosphate buffer and citrate buffer. A particularly preferred pH of the recombinant virus formulation is 7.4, and a preferred buffer is tromethamine.

In addition, it is preferable that the aqueous solution contain a neutral salt which is used to adjust the final formulated recombinant alphavirus to an appropriate iso-osmotic salt concentration. Suitable neutral salts include sodium chloride, potassium chloride or magnesium chloride. A preferred salt is sodium chloride.

Aqueous solutions containing the desired concentration of the components described above may be prepared as concentrated stock solutions.

It will be evident to those skilled in the art, given the disclosure provided herein, that it may be preferable to utilize certain saccharides within the aqueous solution when the lyophilized virus is intended for storage at room temperature. More specifically, it is preferable to utilize disaccharides, such as lactose or trehalose, particularly for storage at room temperature.

The lyophilized or dehydrated viruses of the subject invention may be reconstituted using a variety of substances, but are preferably reconstituted using water. In certain instances, dilute salt solutions which bring the final formulation to isotonicity may also be used. In addition, it may be advantageous to use aqueous solutions containing components known to enhance the activity of the reconstituted virus. Such components include cytokines, such as IL-2, polycations, such as protamine sulfate, or other components which enhance the transduction efficiency of the reconstituted virus. Lyophilized or dehydrated recombinant virus may be reconstituted with any convenient volume of water or the reconstituting agents noted above that allow substantial, and preferably total solubilization of the lyophilized or dehydrated sample.

The following examples are offered by way of illustration, and not by way of limitation.

## EXAMPLES

### Example 1

#### CLONING OF A SINDBIS GENOMIC LENGTH cDNA

The nature of viruses having an RNA genome of positive polarity is such that, when introduced into a eukaryotic cell which serves as a permissive host, the purified genomic nucleic acid serves as a functional message RNA (mRNA) molecule for translation of the viral replicase proteins.

Therefore, this genomic RNA, purified from the virus, can initiate the same infection cycle that is characteristic of infection by the wild-type virus from which the RNA was purified.

For example, Sindbis virus strain AR339 (ATCC #VR-1248, Taylor et al., *Am. J. Trop. Med. Hyg.* 4:844 1955; isolated from the mosquito *Culex univittatus*) is propagated in baby hamster kidney (BHK-21) cells (ATCC #CCL-10), infected at low multiplicity (0.1 PFU/cell). Alternatively, another HR-derived Sindbis virus strain, obtained from Lee Biomolecular (San Diego, Calif.), also is used and propagated by the same methods. Sindbis virions are precipitated from a clarified lysate at 48 hours post-infection, with 10% (w/v) of polyethylene glycol (PEG-

non-viral G ribonucleotide linked to the A ribonucleotide, which corresponds to the authentic Sindbis 5' end. Inclusion of the Apa I recognition sequence facilitates insertion of the PCR amplicon into the plasmid vector (pKS II\*, Stratagene, San Diego, Calif.) polylinker sequence. A five nucleotide 'buffer sequence' is also inserted prior to the Apa I recognition sequence in order to permit efficient digestion. The sequence of the SP6-5' Sindbis forward primer and all of the primer pairs necessary to amplify the entire Sindbis genome are shown below. (Note that "nt" and "nts" as utilized hereinafter refer to "nucleotide" and "nucleotides," respectively). The reference sequence (GenBank accession no. SINCG) is from Strauss et al., *Virology* 133:92-110.

Primer	Location	Seq. ID No.	Sequence	Recognition Sequence (5'→3')
SP6-1A	Apa I/SP6+/SIN nts.1-18	4	TATATGGGCCCGATTAGGTGAC ACTATAGATTGACGGCGTAGTAC AC	Apa I
1B	3182-3160	5	CTGGCAACCGGTAAGTACGATAC	Age I
2A	3144-3164	6	ATACTAGCCACGGCCGGTATC	Age I
2B	5905-5885	7	TCCCTCTTCGACGTGTCGAGC	Eco RI
3A	5844-5864	8	ACCTTGGAGCGCAATGTCTCTG	Eco RI
7349R	7349-7328	9	CCTTTTCAGGGGATCCGCCAC	Bam HI
7328F	7328-7349	10	GTGGCGGATCCCTGAAAAGG	Bam HI
3B	9385-9366	11	TGGGCGGTGTGGTCGTCATG	Bcl I
4A	9336-9356	12	TGGGTCTTCAACTCACCGGAC	Bcl I
10394R	10394-10372	13	CAATTGACGTCACGCTCACTC	Bsi WI
10373F	10373-10394	14	GAGTGAGGCGTACGTCGAATTG	Bsi WI
4B	Xba I/dT <sub>25</sub> /11703-11698	3	TATATTCTAGA(dT) <sub>25</sub> -GAAATG	Xba I

8000) at 0° C., as described previously. Sindbis virions which are contained in the PEG pellet are then lysed with 2% SDS, and the polyadenylated mRNA isolated by chromatography utilizing commercially available oligo-dT columns (Invitrogen, San Diego, Calif.).

Two rounds of first strand cDNA synthesis are performed on the polyA selected mRNA, using an oligonucleotide primer with the sequence shown below:

5'-TATATTCTAGA(dT)<sub>25</sub>-GAAATG-3'(SEQ. ID NO. 3)

Briefly, this primer contains at its 5' end, a five nucleotide 'buffer sequence' for efficient restriction endonuclease digestion, followed by the Xba I recognition sequence, 25 consecutive dT nucleotides and six nucleotides which are precisely complementary to the extreme Sindbis 3' end. Thus, selection for first round cDNA synthesis occurs at two levels: (1) polyadenylated molecules, a prerequisite for functional mRNA, and (2) selective priming from Sindbis mRNA molecules, in a pool possibly containing multiple mRNA species. Further, the reverse transcription is performed in the presence of 10 mM MeHgOH to mitigate the frequency of artificial stops during reverse transcription.

Primary genomic length Sindbis cDNA is then amplified by PCR in six distinct segments using six pairs of overlapping primers. Briefly, in addition to viral complementary sequences, the Sindbis 5' end forward primer is constructed to contain a 19 nucleotide sequence corresponding to the bacterial SP6 RNA polymerase promoter and the Apa I restriction endonuclease recognition sequence linked to its 5' end. The bacterial SP6 RNA polymerase is poised such that transcription in vitro results in the inclusion of only a single

PCR amplification of Sindbis cDNA with the six primer sets shown above is performed in separate reactions, using the THERMALASE™ thermostable DNA polymerase (Amersco Inc., Solon, Ohio) and the buffer containing 1.5 mM MgCl<sub>2</sub>, provided by the supplier. Additionally, the reactions contain 5% DMSO, and the HOT START WAX™ beads (Perkin-Elmer), using the PCR amplification protocol shown below:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	3.5	
72	10	10

Following amplification, the six reaction products are inserted first into the pCR II vector (Invitrogen), then using the appropriate enzymes shown above, are inserted, stepwise, into the pKS II\* (Stratagene) vector, between the Apa I and Xba I sites. This clone is designated the Sindbis genomic cDNA clone pVGSP6GEN is linearized by digestion as pVGSP6GEN.

The Sindbis genomic cDNA clone pVGSP6GEN is linearized by digestion with Xba I, which cuts pVGSP6GEN once, immediately adjacent and downstream of the 25 nucleotide long poly dA:dT stretch. The linearized pVGSP6GEN clone is purified with GENECLEAN™ (BIO 101, La Jolla, Calif.), and adjusted to a concentration of 0.5 mg/ml. Transcription of the linearized pVGSP6GEN clone is performed in vitro at 40° C. for 90 minutes according to the following reaction conditions: 2 ul DNA/4.25 ul H<sub>2</sub>O; 10 ul 2.5 mM NTPs (UTP, ATP, GTP, CTP); 1.25 ul 20 mM

Me<sup>7</sup>G(5')ppp(5')G cap analogue; 1.25 ul 100 mM DTT; 5 ul 5× transcription buffer (Promega, Madison, Wis.); 0.5 ul RNasin (Promega); 0.25 ul 10 mg/ml bovine serum albumin; and 0.5 ul SP6 RNA polymerase (Promega). The in vitro transcription reaction products can be digested with DNase I (Promega) and are purified by sequential phenol/CHCl<sub>3</sub> and ether extraction, followed by ethanol precipitation, or alternatively, can be used directly for transfection. The in vitro transcription reaction products or purified RNA are complexed with a commercial cationic lipid compound (for example, LIPOFECTIN™, GIBCO-BRL, Gaithersburg, Md.), and applied to BHK-21 cells maintained in a 60 mM petri dish at 75% confluency. The transfected cells are incubated at 30° C. After 94 hours post-transfection, extensive cytopathologic effects (CPE) are observed. No obvious CPE is observed in plates not receiving RNA transcribed from the Sindbis cDNA clone. Further, 1 ml of supernatant taken from transfected cells, added to fresh monolayers of BHK-21 cells, and incubated at 30° C. or 37° C. results in obvious CPE within 18 hours. This demonstrates that the Sindbis cDNA clone pVGSP6GEN is indeed infectious.

Sequence analysis of pVGSP6GEN, shown in Table 1, reveals multiple sequence differences between the Sindbis genomic clone described herein, and the viral clone sequence provided in Genbank (GenBank Accession No. SINCG). Many sequence differences result in the substitution of non-conservative amino acids changes in the Sindbis proteins. To address which sequence changes are unique to the virus strain used for cloning, as described herein, or are a result of cloning artifact, virion RNA is amplified by RT-PCR as described above, and sequence relating to the nucleotides in question is determined by direct sequencing of the RT-PCR amplicon product, using a commercially available kit (Promega, Madison Wis.), and compared to the corresponding pVGSP6GEN sequence. The results of this study are given in Table 2. Briefly, three non-conservative amino acid changes, Gly→Glu, Asp→Gly, and Tyr→Cys, which are a result of cloning artifact are observed respectively at viral nucleotides 2245, 6193, and 6730. These nucleotide changes resulting in non-conservative amino acid changes all map to the viral non-structural protein (NSP) genes, nt 2245 to NSP 2, and nts 6193 and 6730 to NSP4.

Repair of the NSP 2 and NSP 4 genes is accomplished by RT-PCR, as described above, using virion RNA from a 5 times plaque purified stock. The SP6-1A/1B primer pair described above is used to repair the nt 2245 change. The RT-PCR amplicon product is digested with Eco 47III and Bgl II, and the 882 bp fragment is purified by 1% agarose/TBE gel electrophoresis, and exchanged into the corresponding region of the pVGSP6GEN clone, prepared by digestion with Eco 47III and Bgl II, and treatment with CIAP. The 3A/7349R primer pair described above is used to repair the nt 6193 and nt 6730 changes. The RT-PCR amplicon product is digested with Eco RI and Hpa I, and the 1,050 bp fragment is purified by 1% agarose/TBE gel electrophoresis, and exchanged into the corresponding region of the pVGSP6GEN clone. This clone is designated pVGSP6GENrep. Transfection of BHK cells with in vitro transcribed RNA from pVGSP6GENrep DNA, linearized by digestion with Xba I as described above, results in extensive CPE within 18 hours post-transfection.

# SINDBIS GENOMIC CLONE DIFFERENCES BETWEEN pVGSP6GEN AND GENBANK SEQUENCES

SIN nt #	Change	Codon Change	Location in Codon	amino acid change
<b>Noncoding Region:</b>				
45	T→C	N.A.	N.A.	N.A.
<b>Non-structural Proteins:</b>				
10	353	C→T	UAU→UAC	3' Tyr→Tyr
	1095	A→C	AUA→CUA	1' Ile→Leu
	1412	T→C	UUU→UUC	3' Phe→Phe
	2032	A→G	GAG→GGG	2' Glu→Gly
15	2245	G→A	GGG→GAG	2' Gly→Glu
	2258	A→C	UCA→UCC	3' Ser→Ser
	2873	A→G	CAA→CAG	3' Gln→Gln
	2992	C→T	CCC→CUC	2' Pro→Leu
	3544	T→C	GUC→GCC	2' Val→Leu
	3579	A→G	AAA→GAA	1' Leu→Glu
20	3822	A→G	ACC→GCC	1' Thr→Ala
	3851	T→C	CUU→CUC	3' Leu→Leu
	5351	A→T	CAA→CAU	3' Gln→His
	5466	G→A	GGU→AGU	1' Gly→Ser
	5495	T→C	AUU→AUC	3' Ile→Ile
	5543	A→T	ACA→ACU	3' Thr→Thr
25	5614	T→C	GUA→GCA	2' Val→Ala
	6193	A→G	GAC→GGC	2' Asp→Gly
	6564	G→A	GCA→ACA	1' Ala→Thr
	6730	A→G	UAC→UGC	2' Tyr→Cys
<b>Structural Proteins:</b>				
30	8637	A→G	AUU→GUU	1' Ile→Val
	8698	T→A	GUA→GAA	2' Val→Glu
	9108	AAG del	AAG→del	1'-3' Glu→del
	9144	A→G	AGA→GGA	1' Arg→Gly
	9420	A→G	AGU→GGU	1' Ser→Gly
35	9983	T→G	GCU→GCG	3' Ala→Ala
	10469	T→A	AUU→AUA	3' Ile→Ile
	10664	T→C	UUU→UUC	3' Phe→Phe
	10773	T→G	UCA→GCA	1' Ser→Ala

TABLE 2

<b>SINDBIS GENOMIC CLONE ARTIFACT ANALYSIS</b>			
SIN nt #	Amino Acid change	pVGSP6GEN Unique	Cloning Artifact
<b>Nonstructural Proteins:</b>			
2032	Glu→Gly	+	
2245	Gly→Glu		+
2258	Ser→Ser	+	
2873	Gln→Gln		
2992	Pro→Leu	+	
3544	Val→Leu		+
3579	Leu→Glu	+	
3822	Thr→Ala		+
3851	Leu→Leu		+
5351	Gln→His	+	
5466	Gly→Ser		+
5495	Ile→Ile		+
5543	Thr→Thr		+
6193	Asp→Gly		+
6730	Tyr→Cys		
<b>Structural Proteins:</b>			
8637	Ile→Val	+	
8698	Val→Glu	+	
9108	Glu→del	+	
9144	Arg→Gly	+	

\*Mixture: Both Genbank and pVGSP6GEN Sindbis strains present at this nucleotide.

# GENERATION OF DNA VECTORS WHICH INITIATE ALPHAVIRUS INFECTION: EUKARYOTIC LAYERED VECTOR INITIATION SYSTEMS

As noted above, the present invention provides eukaryotic layered vector initiation systems which generally comprise a promoter which is capable of initiating the 5' synthesis of RNA from cDNA, a construct which is capable of autonomous or autocatalytic replication in a cell, the construct also being capable of expressing a heterologous nucleic acid sequence, and a 3' sequence which controls transcription termination. Within one embodiment, such constructs may be constructed of the following ordered elements: a 5' eukaryotic promoter capable of initiating the synthesis of viral RNA at the authentic alphavirus 5' end, a 5' sequence which is capable of initiating transcription of an alphavirus, a nucleotide sequence encoding alphavirus non-structural proteins, a viral junction region, a heterologous sequence, an alphavirus RNA polymerase recognition sequence, and a 3' transcription termination/polyadenylation signal sequence. Such alphavirus cDNA expression vectors may also include intervening sequences (introns), which are spliced from the pre-RNA in the nucleus prior to transport to the cytoplasm, and which may improve the overall efficiency of the system, in terms of molecules of functional mRNA transported to the cytoplasm/nuclear DNA template. The intron splicing signals are located, for example, between Sindbis and heterologous gene regions as described in Example 3.

Construction of a eukaryotic layered vector initiation system utilizing the Sindbis clone pVSP6GENrep and mammalian RNA polymerase II promoters is accomplished as follows. Briefly, plasmid pVSP6GENrep is digested with Bgl II and Xba I, and the reaction products are electrophoresed on a 0.8% agarose/TBE gel. The resulting 9,438 bp fragment is excised, purified with GENECLEAN™, and ligated into the 4,475 bp vector fragment resulting from treatment of pCDNA3 (Invitrogen) with Bgl II, Xba I, and CIAP. This construction is designated as pcDNASINbgl/xba.

The U3 region of the long terminal repeat (LTR) from Moloney murine leukemia virus (Mo-MLV) is positioned at the 5' viral end such that the first transcribed nucleotide is a single G residue, which is capped in vivo, followed by the Sindbis 5' end. Juxtaposition of the Mo-MLV LTR and the Sindbis 5' end is accomplished by overlapping PCR as described below. Amplification of the Mo-MLV LTR in the first primary PCR reaction is accomplished in a reaction containing the BAG vector (Price et al., *PNAS* 84:156-160, 1987) and the following primer pair:

Forward primer: BAGBgl2F1 (buffer sequence/Bgl II recognition sequence/Mo-MLV LTR nts 1-22):

5'-TATATAGATCTAATGAAAGACCCACCTGTAGG (SEQ. ID NO. 15)

Reverse primer: BAGwt441R2 (SIN nts 5-1/Mo-MLV LTR nts 441-406):

5'-TCAATCCCCGAGTGAGGGGTTGTGGGCTCTTT-ATTGAGC (SEQ. ID NO. 16)

PCR amplification of the Mo-MLV LTR with the primer pair shown above is performed using the THERMALASE™ thermostable DNA polymerase and the buffer containing 1.5 mM MgCl<sub>2</sub>, provided by the supplier. Additionally, the reaction contains 5% DMSO, and the HOT START WAX™ beads, using the PCR amplification protocol shown below:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	0.5	
72	10	1

Amplification of the Sindbis 5' end in the second primary PCR reaction is accomplished in a reaction containing the pVSP6GENrep clone and the following primer pair:

Forward primer: (Mo-MLV LTR nts 421-441/SIN nts 1-16):

5'-CCACAACCCCTCACTCGGGGATTGACG-GCGTAGTAC (SEQ. ID NO. 17)

Reverse primer: (SIN nts 3182-3160):

5'-CTGGCAACCGGTAAGTACGATAC (SEQ. ID NO. 18)

PCR amplification of the Mo-MLV LTR is accomplished with the primer pair and amplification reaction conditions described above, utilizing the PCR amplification protocol shown below:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	3.0	
72	10	1

The 457 bp and 3202 bp products from the primary PCR reactions are GENECLEAN™, and combined in a secondary PCR reaction with the following primer pair:

Forward primer: BAGBgl2F1 (buffer sequence/Bgl II recognition sequence/Mo-MLV LTR nts 1-22):

5'-TATATAGATCTAATGAAAGACCCACCTGTAGG (SEQ ID NO. 15)

Reverse primer: (SIN nts 2300-2278):

5'-GGTAACAAGATCTCGTGCCGTG (SEQ ID NO. 19)

PCR amplification of the primer PCR amplicon products is accomplished utilizing the primer pair and amplification reaction conditions shown above, and using the following PCR amplification protocol:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	3.0	
72	10	1

The 25 3' terminal bases of the first primary PCR amplicon product overlaps with the 25 5' terminal bases of the second primary PCR amplicon product; the resultant 2,752 bp overlapping secondary PCR amplicon product is purified by 0.8% agarose/TBE electrophoresis, digested with Bgl II, and the 2,734 bp product is ligated into pcDNASINbgl/xba treated with Bgl II and CIAP. The resulting construction is 16,656 bps and is designated pVGELVIS. The sequence of pVGELVIS is given in FIG. 3 (SEQ. ID NO. 1) Sindbis nucleotides are contained within bases 1-11,700 of the sequence.

pVGELVIS plasmid DNA is complexed with LIPO-FECTAMINE™ (GIBCO-BRL, Gaithersburg, Md.) accord-

ing to the conditions suggested by the supplier (ca. 5 ug DNA/8 ug lipid reagent) and added to 35 mm wells containing BHK-21 cells at approximately 75% confluency. Cytopathic effects (CPE), characteristic of wild type Sindbis virus infection are observed within 48 hours post-infection. Addition of 1 ml of transfection supernatant to fresh BHK-21 monolayers results in CPE within 16 hrs. This data demonstrates the correct juxtaposition of viral cDNA and RNA polymerase II expression cassette signals in the pVGELVIS construct, resulting in the de novo initiation of an RNA virus from a DNA expression module.

In order to determine the relative efficiency of the pVGELVIS plasmid DNA to initiate infection characteristic of wild type Sindbis virus after transfection into BHK cells, an infectious centers assay is performed. Briefly, 5 ug of pVGELVIS plasmid DNA is transfected into BHK-21 cells in 35 mm wells as described above, and at 1.5 hours post transfection the cells are trypsinized and serially diluted 10,000-fold, over 10-fold increments, into  $5 \times 10^5$  untreated BHK cells. This transfected and untreated BHK cell mixture is then added to 35 mm wells. The cells are allowed to attach to the plate, and subsequently overlaid with media containing 1.0% Noble Agar. At 48 hrs post transfection, plaques due to cell lysis (as a result of Sindbis virus replication) are visualized either directly or after overlaying with a second layer containing Neutral Red Stain. This experiment reveals that the efficiency of the pVGELVIS plasmid in generating wild type Sindbis virus after transfection onto BHK cells is approximately  $1 \times 10^4$  PFU/ug of plasmid DNA.

#### Example 3

##### PREPARATION OF RNA AND DNA ALPHAVIRUS VECTORS

###### A. CONSTRUCTION OF THE SINDBIS BASIC VECTOR

A first step in the construction of the Sindbis Basic Vector is the generation of two plasmid subclones containing separate elements from the viral 5' and 3' ends. These elements may then be utilized in order to subsequently assemble a basic gene transfer vector.

Briefly, the first plasmid subclone is constructed to contain the 40 terminal nucleotides of the viral 3' end and a 25 base pair stretch of consecutive dA:dT nucleotides. In particular, the following oligonucleotide pairs are first synthesized:

Forward Primer: SIN 11664F: (buffer sequence/Not I site/SIN nts 11664-11698):

5'-TATATATATATGCGGCCGCTTTCTTTTATTAA-TCAACAAAATTTTGTTTTAA (SEQ. ID NO. 20)

Reverse Primer: SIN Sac 11700R (buffer sequence/Sac I site dT25/SIN nts 11700-11692):

5'-TATATGAGCTCTTTTCTTTTCTTTTCTTTTCTTTTCTTTTGTAAATGTTAAAA (SEQ. ID NO. 21)

The above oligonucleotides are then mixed together at equal molar concentrations in the presence of 10 mM  $MgCl_2$ , heated to 100° C. for 5 minutes and cooled slowly to room temperature. The partially double-stranded molecule is then filled in using Klenow DNA polymerase and 50 uM dNTPs. The resultant 89 bp molecule is then digested with Not I and Sac I, purified on a 2% NuSieve/1% agarose gel, and ligated into pKS II+ plasmid (Stratagene, La Jolla, Calif.), prepared by digestion with Not I and Sac I and treatment with CIAP, at a 10:1 molar excess of insert:vector ratio. This construction is designated pKSII3'SIN.

The second plasmid subclone is constructed to contain the first 5' 7,643 nucleotides of Sindbis, and a bacteriophage

RNA polymerase promoter is positioned at the viral 5' end such that only a single non-viral nucleotide is added to the authentic viral 5' end after in vitro transcription. Briefly, the 3' end of this clone is derived by a standard three temperature PCR amplification with a reverse primer having the sequence shown below.

Reverse Primer: SINXho7643R (buffer sequence/Xho I site/SIN nts 7643-7621):

5'TATATCTCGAGGGTGGTGTGTAGTATTAGTCAG (SEQ. ID NO. 22)

The reverse primer maps to viral nucleotides 7643-7621 and is 41 bp downstream from the junction core element 3' end. Additionally, viral nucleotide 7643 is 4 nucleotides upstream from the structural protein gene translation initiation codon. The first five 5' nucleotides in this primer are included to serve as a 'buffer sequence' for the efficient digestion of the PCR amplicon products, and are followed by 6 nucleotides comprising the Xho I recognition sequence.

The forward primer in this reaction is primer 2A (described in Example 1), having the following sequence:

ATACTAGCCACGGCCGGTATC (SEQ. ID NO. 6)

The 4510 bp amplicon product, resulting from the PCR amplification shown above with pVGSP6GENrep plasmid (described in Example 1) as template, is digested with the enzymes Sfi I and Xho I. The resultant 2526 bp fragment is gel purified. Sindbis cDNA clone pVGSP6GENrep is also digested with Apa I and Sfi I, and the resultant 5144 bp fragment which includes the SP6 RNA polymerase promoter at its 5' end is gel purified. The 5144 bp fragment is ligated together with the 2526 bp fragment from above, along with Apa I and the Xho I digested CIAP treated pKS II+ plasmid. A clone is isolated having the Sindbis nucleotides 1-7643 including the RNA polymerase promoter at its 5' end contained in the pKSII+ plasmid vector. This construction is designated pKSII5'SIN.

Assembly of the complete basic vector is accomplished by digesting pKSII5'SIN with Xho I and Sac I, treating with CIAP, and gel purifying of a large 10,533 bp fragment. The 10,533 bp fragment is then ligated together with a 168 bp small fragment resulting from digestion of pKSII3'SIN with Xho I and Sac I. This resultant construction is designated pKSSINBV (also known as SINDBIS basic vector, see FIG. 4).

###### B. CONSTRUCTION OF SINDBIS LUCIFERASE VECTOR

The firefly luciferase reporter gene is inserted into the Sindbis Basic Vector in order to demonstrate the expression of a heterologous gene in cells transfected with RNA that is transcribed in vitro from the Sindbis vector clone, and to demonstrate the overall functionality of the Sindbis basic vector.

Construction of the Sindbis luciferase vector is performed by assembling together components of 3 independent plasmids: pKSII5'SIN, pKSII3'SIN, and pGL2-basic vector. The pGL2-basic vector plasmid (Promega, Madison, Wis.) contains the entire firefly luciferase gene. Briefly, the luciferase gene is first inserted into the pKSII3'SIN plasmid. This is accomplished by digesting pGL2 with Bam HI and Hind III, and gel purifying a 2689 bp containing fragment. This fragment is ligated with a gel purified 3008 bp large fragment resulting from digestion of pKSII3'SIN with Bam HI and Hind III and treatment with CIAP. The resultant construction is designated pKSII3'SIN-luc.

Final assembly of a Sindbis luciferase vector is accomplished by digesting pKSII5'SIN with Xho I and Sac I, treating with CIAP, and gel purifying the large 10,533 bp fragment. The pKS 5'SIN 10,533 bp fragment is ligated



together with the 2854 bp small fragment resulting from digestion of pKSI3'SIN-luc with Xho I and Sac I. This construction contains the entire Sindbis nonstructural gene coding region and 3' viral elements necessary for genome replication, as well as the firefly luciferase gene positioned between these two viral 5' and 3' elements. This vector is designated pKSSINBV-luc (also known as SINDBIS-luciferase) and is shown schematically in FIG. 4.

#### C. EXPRESSION OF LUCIFERASE IN TRANSFECTED AND INFECTED BHK-21 CELLS

In order to test the functionality of the Sindbis Basic Vector, the expression of luciferase in cells transfected with RNA transcribed in vitro from Sac I-linearized pKSSINBV-luc, as described in Example 1, is tested.

In addition, a complementary packaging vector, which is deleted of most of the non structural gene region, is constructed by digestion of pVGSP6GENrep with Bsp EI and re-ligation under dilute conditions. This construction, designated pVGSP6GENd1Bsp (also known as "dl Bsp EI") is deleted of nonstructural gene sequences between bases 422-7,054, and is shown schematically in FIG. 5. Transcription in vitro of Xba I-linearized pVGSP6GENd1Bsp is as described in Example 1. Transfections and co-transfections are performed by complexing in vitro transcription products with LIPOFECTIN™ and applying to BHK-21 cells. The expression of luciferase in transfected cells is tested 18 hours after transfection. Additionally, 1 ml of the transfection supernatant is used to infect a confluent monolayer of BHK-21 cells and the expression of luciferase is tested at 24 hours post-infection.

The results of this experiment shown in FIG. 6, demonstrate clearly abundant reporter gene expression follows transfection of BHK-21 cells with in vitro transcribed RNA from pKSSINBV-luc, and transfer (e.g., packaging) of the expression activity when cells are co-transfected with in vitro transcribed RNA from pVGSP6GENd1Bsp.

#### D. CONSTRUCTION OF ALTERED JUNCTION REGION SINDBIS VECTORS

In order to inactivate the Sindbis viral junction region, nucleotides within the NSP4 carboxy terminus and junction region overlap are changed, and the vector nucleotides corresponding to Sindbis are terminated prior to the subgenomic initiation point at Sindbis nt 7598. This construction is shown schematically in FIG. 7.

Briefly, a fragment is PCR amplified from the pKSSINBV clone under nonstringent reaction cycle conditions utilizing a reverse primer having the following sequence:

TATATGGGCCCCTTAAGACCATCGGAG  
CGATGCTTTATTTCCCC (SEQ. ID NO. 23)

The underlined bases in the reverse primer relate to nucleotide changes which can be made in the junction region without affecting the coded amino acid (see below). All of the nucleotide changes are transversions.

3' end of NSP 4 (viral nts 7580-7597).

TCT	CTA	CGG	TGG	TCC	TAA	(SEQ. ID NO. 24)
ser	leu	arg	trp	ser	stop	(SEQ. ID NO. 25)
G	C	A	T			

(resulting nt changes from reverse primer)

The reverse primer is complementary to Sindbis nts 7597-7566 (except at nucleotides, as shown, where junction region changes were made), and includes at its 5' end the 6 nucleotide Apa I recognition sequence following a 5' terminal TATAT tail 'buffer sequence' for efficient enzyme digestion.

The forward primer in this reaction is primer 2A (described in Example 1), having the following sequence:

5'-ATACTAGCCACGGCCGGTATC (SEQ. ID NO. 6)

The 4,464 bp amplicon resulting from a PCR reaction with pKSSINBV template and using the primer pair described above is digested with Sfi I and Apa I and the gel purified 2,480 bp fragment is ligated together with the gel purified 5,142 bp fragment resulting from the digestion of pKSSINBV with Apa I and Sfi I, and with the gel purified 2,961 bp fragment resulting from the digestion of pKSI3'SIN with Apa I and from the treatment with CIAP. This construction, comprised of Sindbis nucleotides 1-7597, including the changes in the junction region described above, and including the bacterial SP6 promoter attached to Sindbis nt 1 is referred to as pKSS'SINdIJR.

Final construction of the inactivated junction region vector is accomplished by ligation of the 7,622 bp large Sindbis fragment resulting from digestion of pKSS'SINdIJR with Apa I, with the 3,038 bp fragment resulting from digestion of pKSI3'SIN with Apa I and treatment with CIAP. The positive orientation of the 5' Sindbis element, relative to the 3' Sindbis element, is confirmed by restriction endonuclease analysis. This construction is referred to as pKSSINBVdIJR.

Initiation and synthesis of subgenomic mRNA cannot occur from the pKSSINBVdIJR vector. In order to prove this supposition, comparative RNase protection assays using the pKSSINBV and pKSSINBVdIJR vectors are performed. Briefly, a <sup>32</sup>P-end labeled RNA probe complementary in part to the junction region, including the subgenomic RNA initiation point at viral nt 7,598 is used to hybridize with the viral RNA resulting from the transfection of BHK-21 cells with the pKSSINBV and pKSSINBVdIJR vectors. The RNase protection assay demonstrates that cells transfected with pKSSINBV have two fragments, of genomic and subgenomic specificity, while cells transfected with pKSSINBVdIJR have only a single fragment of genomic specificity. These results prove that the junction region in the pKSSINBVdIJR vector is indeed inactivated.

In order to test translation of genomic RNA from the region corresponding to the subgenomic RNA message, the luciferase reporter gene is inserted into the inactivated junction region vector pKSSINBVdIJR described above. This construction is accomplished by digesting the pKSSINBVdIJR with Xho I and Sac I, treating with CIAP, and gel purifying the resulting 10,197 bp fragment. The pKSSINBVdIJR fragment is ligated together with the 2854 bp small fragment resulting from digestion of pKSI3'SIN-luc with Xho I and Sac I. This construction contains the entire Sindbis nonstructural gene coding region terminating in an inactivated junction region at Sindbis nt 7597, and 3' viral elements necessary for genome replication; the firefly luciferase gene is placed between these two viral 5' and 3' elements. This vector is known as pKSSINBVdIJR-luc.

The expression of the reporter gene from the pKSSINBVdIJR-luc vector is tested in transfected BHK-21 cells. Translation of functional luciferase protein is determined by the luciferin luminescent assay, using a luminometer for detection. The sensitivity in this assay is  $1 \times 10^{-20}$  moles of luciferase. Given that the molecular weight of luciferase is 62,000 daltons, this limit of detection transforms to 6,020 molecules. Thus, in a typical experiment if only 0.6% of the  $1 \times 10^6$  cells contained in a 60 mM petri dish are transfected with the pKSSINBVdIJR-luc vector, and if these transfected cells express only a single functional molecule of luciferase, the enzymatic activity is detected by the assay used. It is important to demonstrate in this experiment that the junction region of the pKSSINBVdIJR-luc vector is inactivated. This is accomplished by an RNase protection assay, comparing the viral RNA's synthesized in

cells transfected with the pKSSINBVdJLR-luc and the pKSSINBV-luc vectors, using the probe described above.

The minimal -19→+5 junction region core oligonucleotide pair, comprised of Sindbis nts 7579-7602, is synthesized in vitro, and flanked with Apa I and Xho I recognition sequences as shown:

oligonucleotide 1

5'-CATCTCTACGGTGGTCCTAAATAGTC (SEQ. ID NO. 26)

oligonucleotide 2

5'-TCGAGACTATTTAGGACCACCGTAGAGATGGGCC (SEQ. ID NO. 27)

The oligonucleotides above are mixed together in the presence of 10 mM Mg<sup>2+</sup>, heated to 100° C. for 5 minutes and cooled slowly to room temperature. The annealed oligonucleotides are ligated at a 25:1 molar ratio of insert to the pKSSINBVdJLR vector, prepared accordingly: complete digestion with Xho I, followed by digestion with Apa I under partial conditions, resulting in one Apa I induced cleavage per molecule (of two cleavages possible), gel purification of the 10,655 bp fragment, and treatment with CIAP. This vector containing the entire nonstructural protein coding region which terminates in an inactivated junction region core, attached to a synthetic junction region core and followed by 3' viral elements required for replication, and contained in the pKSI+ plasmid, is known pKSSINdJLRsjrc.

In order to regulate the level of subgenomic mRNA synthesis, further modifications of the tandemly inserted synthetic junction region core in plasmid pKSSINdJLRsjrc are performed. These modifications of the junction region core may be accomplished by at least two approaches: nucleotide changes within the junction region core; or extension at the 5' and 3' junction region core termini of flanking Sindbis nucleotides, according to authentic viral sequence. The minimal junction region core, spanning viral nts 7579-7602 is shown below:

5'-ATCTCTACGGTGGTCCTAAATAGT (SEQ. ID NO. 2)

By comparing genomic sequence between eight alphaviruses, it has been shown previously that there is sequence diversity within the junction region core. Shown below, for particular junction region locations, is the Sindbis nucleotide followed by the corresponding nucleotide found in other alphaviruses:

Nucleotide Number	Sindbis	Permissive Change
7579	A	C
7580	U	C
7581	C	U
7583	C	G
7589	U	C
7590	G	U
7591	G	A
7592	U	A
7600	A	U or G
7602	U	G or A

Junction region changes at Sindbis nts 7579, 7580, 7581, 7583, 7589, 7590, 7591, 7592, result in potential amino acid coding changes within all 5 codons of the carboxy terminus of NSP 4 which overlap in the junction region. These changes observed in the junction region between alphaviruses at the level of NSP 4 coding potential and at the level of junction region cis activity may represent either, or both, permissive changes in NSP 4 and the junction region which do not affect functionality, or on the other hand, simply

different viruses. In any event, the junction region changes presented herein regard the tandemly inserted junction region core, from which no NSP protein synthesis occurs. Discussed above, translation of the entire NSP region occurs from the pKSSINBVdJLR construct. Junction region changes at Sindbis nts 7600 and 7602 are downstream of the NSP 4 termination codon and upstream of the structural proteins initiation codon.

Locations of nucleotide differences within the junction region core observed between the several alphavirus strains are referred to here as permissive changes. Locations of nucleotides within the junction region core corresponding to conserved sequences between the several alphavirus strains are referred to here as nonpermissive changes.

To decrease the level of subgenomic mRNA initiation from the synthetic junction region core, changes are made separately within nucleotides corresponding to permissive changes, and within nucleotides corresponding to nonpermissive changes. Junction region nucleotides corresponding to permissive changes are given in the table above. Fourteen junction region nucleotides for which no changes are observed among the eight alphaviruses sequenced (Semliki Forest virus, Middleburg virus, Ross River virus, O'Nyong Nyong virus, Eastern Equine Encephalitis virus, Western Equine Encephalitis virus, and Venezuelan Equine Encephalitis virus) are given below:

Nucleotide Number:
7582
7584
7585
7586
7587
7588
7593
7594
7595
7596
7597
7598
7599
7601

Changes within the junction region observed among alphaviruses may reflect a specific interaction between a given alphaviral RNA polymerase and its cognate junction region. Thus, changes among the "permissive" nucleotides may result in as marked a decrease in the subgenomic mRNA synthesis levels as changes among the "nonpermissive" nucleotides of the junction region. On the other hand, these may indeed be sites of permissive change within the junction region core.

The single authentic nonpermissive change within the junction region core is likely Sindbis nt 7598, corresponding to the subgenomic mRNA initiation point. Changes of this nucleotide in the tandemly inserted junction region core of plasmid pKSSINdJLRsjrc are not described here.

Substitution of the permissive nucleotides in toto in the synthetic minimal -19→+5 junction region core, is accomplished with the following oligonucleotide pair, synthesized in vitro, and flanked with Apa I and Xho I recognition sequences as shown:

oligonucleotide 1

5'-CCCTTGTACGGCTAACCTAAAGGAC (SEQ. ID NO.28)

oligonucleotide 2

5'-TCGAGTCCTnTAGGTTAGCCGTACAAGGGGGGCC (SEQ. ID NO.29)

The oligonucleotides above are mixed together in the presence of 10 mM Mg, heated to 100° C. for 5 minutes and cooled slowly to room temperature. The annealed oligonucleotides are ligated at a 25:1 molar ratio of insert to the pKSSINBVdJR vector, prepared accordingly: complete digestion with Xho I, followed by digestion with Apa I under partial conditions, resulting in one Apa I induced cleavage per molecule (of two cleavages possible), gel purification of the 10,655 bp fragment, and treatment with CIAP. This vector is known as pKSSINdJRsjrPc.

Each of the 13 (nt 7598 not changed) nonpermissive nucleotides in the junction region core are changed individually, using the following rules, resulting in the most drastic transversional substitution:

A→C

T→G

G→T

C→A

For example, nt 7582 is changed from T→G, using the following oligonucleotide pair, synthesized in vitro, and flanked with Apa I and Xho I recognition sequences as shown:

oligonucleotide 1

5'-CATCGCTACGGTGGTCCTAAATAGTC (SEQ. ID NO. 30)

oligonucleotide 2

5'-TCGAGACTATTTAGGACCACCGTAGCGATGGGCC (SEQ. ID NO. 31)

(Nucleotides effecting transversion in nonpermissive junction region sites shown in boldface type)

The oligonucleotides above are mixed together in the presence of 10 mM Mg<sup>2+</sup>, heated to 100° C. for 5 minutes and cooled slowly to room temperature. The annealed oligonucleotides are ligated at a 25:1 molar ratio of insert to the pKSSINBVdJR vector, prepared accordingly: complete digestion with Xho I, followed by digestion with Apa I under partial conditions, resulting in one Apa I induced cleavage per molecule (of two cleavages possible), gel purification of the 10,655 bp fragment, and treatment with CIAP. This vector is known as pKSSINdJRsjrNP7582.

Using the transversion change rules shown above, changes in each of the 12 remaining nonpermissive sites in the junction region core are made with 12 separate oligonucleotide pairs, flanked with Apa I and Xho I recognition sites, as described above. These vectors are known as:

pKSSINdJRsjrNP7584

pKSSINdJRsjrNP7585

pKSSINdJRsjrNP7586

pKSSINdJRsjrNP7587

pKSSINdJRsjrNP7588

pKSSINdJRsjrNP7593

pKSSINdJRsjrNP7594

pKSSINdJRsjrNP7595

pKSSINdJRsjrNP7596

pKSSINdJRsjrNP7597

pKSSINdJRsjrNP7599

pKSSINdJRsjrNP7601

In order to test the relative levels of subgenomic mRNA synthesis, the luciferase reporter gene is inserted into the modified tandem junction region vectors. This construction is accomplished by digesting with Xho I and Sac I and treating with CIAP the tandemly inserted synthetic junction region core vectors and gel purifying the resulting approximate 10,200 bp fragment. The treated vector fragment is

then ligated together with the 2854 bp small fragment resulting from digestion of pKSI3'SIN-luc with Xho I and Sac I. These constructions contain the entire Sindbis non-structural gene coding region terminating in an inactivated junction region at Sindbis nt 7597, the tandemly inserted synthetic junction region core (modified or unmodified), the firefly luciferase gene, and 3' viral elements necessary for genome replication. The names of these vectors are as follows:

Sindbis-luciferase vector	Tandemly Inserted Junction Region Modification
pKSSINdJRsjr-luc	not modified
pKSSINdJRsjrPc-luc	permissive changes
pKSSINdJRsjrNP7582-luc	nonpermissive change
pKSSINdJRsjrNP7584-luc	.
pKSSINdJRsjrNP7585-luc	.
pKSSINdJRsjrNP7586-luc	.
pKSSINdJRsjrNP7587-luc	.
pKSSINdJRsjrNP7588-luc	.
pKSSINdJRsjrNP7593-luc	.
pKSSINdJRsjrNP7594-luc	.
pKSSINdJRsjrNP7595-luc	.
pKSSINdJRsjrNP7596-luc	.
pKSSINdJRsjrNP7597-luc	.
pKSSINdJRsjrNP7599-luc	.
pKSSINdJRsjrNP7601-luc	.

Assuming that the translation efficiencies are equivalent in all of the luciferase vectors shown immediately above, the relative levels of subgenomic synthesis are determined by comparing the levels of luciferase production at 16 hours post-transfection of BHK-21 cells. The relative levels of subgenomic transcription are determined by comparing luciferase production by the vectors pKSSINBV-luc and pKSSINdJRsjr-luc with all of the modified junction region luciferase vectors shown above.

Vectors containing the tandemly inserted synthetic junction region core (pKSSINdJRsjr, and derivatives thereof) should have a lower level of subgenomic mRNA expression, relative to the pKSSINBV construct. Therefore, in certain embodiments, it may be necessary to increase the level of subgenomic mRNA expression observed from the pKSSINdJRsjr vector. This may be accomplished by extension at the 5' and 3' synthetic junction region core termini with 11 additional flanking Sindbis nucleotides, according to the authentic viral sequence.

The synthetic oligonucleotide pair shown below is synthesized in vitro, and contains 46 Sindbis nts, including all 24 nts (shown in boldface type) of the minimal junction region core. The Sindbis nts are flanked with the Apa I and Xho I recognition sequences as shown:

oligonucleotide 1

5'-CGGAAATAAAGCATCTCTACGGTGGTCCTAAATAGTCAGCATAGTACC (SEQ. ID NO. 32)

oligonucleotide 2

5'-TCGAGGTAATGCTGACTATTTAGGACCACCGTAGAGATGCTTTA TTCCGGGCC (SEQ. ID NO. 33)

The oligonucleotides above are mixed together in the presence of 10 mM Mg, heated to 100° C. for 5 minutes and cooled slowly to room temperature. The annealed oligonucleotides are ligated at a 25:1 molar ratio of insert to the pKSSINBVdJR vector, prepared accordingly: complete digestion with Xho I, followed by digestion with Apa I under partial conditions, resulting in one Apa I induced cleavage per molecule (of two cleavages possible), gel purification of the 10,655 bp fragment, and treatment with CIAP. This

vector containing the entire nonstructural protein coding region which terminates in an inactivated junction region core, attached to an extended synthetic junction region, and followed by 3' viral elements required for replication, and contained in the pKSI+ plasmid, is known pKSSINDJRsexjr.

In order to test the relative levels of subgenomic mRNA synthesis, the luciferase reporter gene is inserted into the extended tandem junction region pKSSINDJRsexjr vector. This construction is accomplished by digesting the pKSSINDJRsexjr plasmid with Xho I and Sac I, treating with CIAP, and gel purifying the resulting approximate 10,200 bp fragment. The thus-treated vector fragment is ligated together with the 2854 bp small fragment resulting from digestion of pKSI3'SIN-luc with Xho I and Sac I. This construction contains the entire Sindbis nonstructural gene coding region terminating in an inactivated junction region at Sindbis nt 7597, the tandemly inserted extended synthetic junction region, the firefly luciferase gene, and 3' viral elements necessary for genome replication. The name of this vector is pKSSINDJRsexjr-luc.

The relative levels of subgenomic transcription are determined by comparing luciferase production by the pKSSINDJRsexjr-luc vector with the pKSSINBV-luc and pKSSINDJRsrc-luc vectors.

#### E. CONSTRUCTION OF PLASMID DNA ALPHAVIRUS EXPRESSION VECTORS

The SINDBIS basic vector and SINDBIS-luciferase constructs described in sections A and B of Example 3, above, are inserted into the pVGELVIS vector configurations described previously in Example 2 such that expression of the heterologous gene from Sindbis vectors occurs after direct introduction of the plasmid DNA into cells. As described in Example 2, the ability to transfect alphavirus-based vector plasmid DNA directly onto cells resulting in expression levels of heterologous genes typical of transfection of RNA-based alphavirus vectors, without a primary step consisting of in vitro transcription of linearized template vector DNA, enhances greatly the utility and efficiency of certain embodiments of the alphavirus-based expression vector system. FIG. 8 is a schematic representation of one mechanism of expression of heterologous genes from a plasmid DNA alphavirus expression (ELVIS) vectors. Primary transcription in the nucleus and transport of the vector RNA to the cytoplasm leads to the synthesis of alphavirus nonstructural proteins which catalyze the expansion of heterologous gene mRNA via an antigenome intermediate which in turn serves as the template for production of genomic and subgenomic mRNA. The ELVIS vectors may be introduced into the target cells directly by physical means as a DNA molecule, as a complex with various liposome formulations, or as a DNA ligand complex including the alphavirus DNA vector molecule, a polycation compound such as polylysine, a receptor specific ligand, and, optionally, a psoralen inactivated virus such as Sendai or Adenovirus.

The first step of constructing one representative plasmid DNA Sindbis expression vector consists of digesting pKSSINBV with Sac I, blunting with T4 polymerase, digesting with Sfi I, isolating the 2,689 bp fragment, and ligating into the pVGELVIS 10,053 bp vector fragment prepared by digestion with XbaI, blunting with T4 polymerase, digesting with Sfi I, treatment with CIAP, and 1% agarose/TBE gel electrophoresis. This construction is known as pVGELVIS-SINBV.

In order to insert the luciferase gene into the pVGELVIS-SINBV vector, the SV40 intron and transcription termina-

tion sequences at the 3'-end of luciferase must be removed so that when the pre-RNA, transcribed from the plasmid DNA luciferase vector after transfection into cells, is processed the 3'-end of the reporter gene is not separated from the Sindbis vector 3'-end. The Sindbis 5'- and 3'-ends contained within the pVGELVIS-SINBV vector are required in cis for the autocatalytic replication activity of the vector. The Sindbis vector 3'-end is required for initiation of synthesis of the antigenomic strand, which is the template for the subgenomic RNA encoding the heterologous or reporter protein.

The SV40 RNA processing signals positioned at the 3'-end of the luciferase gene are removed from the SIN-BV-luc construction described in section B above. The modified luciferase fragment is then placed in the pVGELVIS-SINBV construction described above via unique restriction sites. The alteration of the luciferase gene is accomplished with the primer pair shown below:

Forward primer 7328F (SIN nts 7328-7349)

5'-GTGGCGGATCCCCTGAAAAGG (SEQ. ID NO. 10)

Reverse primer LucStop (buffer sequence/Not I. Xba I recognition sequences/pGL-2 nts 1725-1703)

5'-TATATGCGGCCGCTCTAGATTACAATTTGG-  
ACTTTCGCC (SEQ. ID NO. 34)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period. The amplification products are purified with GENECLEAN™, digested with Xho I and Xba I, purified again with GENECLEAN™, and the 2,037 bp fragment is ligated into the 13,799 bp fragment of pVGELVIS-SINBV resulting from digestion with Xho I and Xba I, and treatment with CIAP. This construction is known as pVGELVIS-SINBV-luc (abbreviated as ELVIS-luc).

The expression of luciferase in BHK-21 cells transfected with pVGELVIS-SINBV-luc DNA is measured in order to demonstrate that the Sindbis physical gene transfer vector is functional. Briefly, 5 ug of pVGELVIS-SINBV-luc DNA or 5 ug of in vitro transcribed RNA from linearized SINBV-luc template as described in section B, above, are complexed with 10 ul of LIPOFECTAMINE™ or LIPOFECTIN™, respectively, and transfected into 5x10<sup>5</sup> BHK-21 cells contained in 35 mM petri plates. The luciferase activity is determined from each of three samples at 2, 4, 8, 16, 20, 28, 48, 72, 96, and 120 hrs. post transfection. The results of this study, given in FIG. 9, demonstrate that the maximal level of reporter gene expression from the pVGELVIS-SINBV-luc vector is similar to that observed in cells transfected with in vitro transcribed RNA from linearized SINBV-luc template. However, the luciferase activity expressed from the pVGELVIS-SINBV-luc vector is at maximal levels at later time points compared to that observed with the SINBV-luc RNA vector, and continues at high levels while the activity from the RNA vector begins to diminish.

The following experiment is performed in order to demonstrate the level of enhancement of heterologous gene expression provided by the ELVIS vector system compared to the same RNA polymerase II promoter linked directly to the luciferase gene reporter. Briefly, the Sindbis NSPs are first deleted from the pVGELVIS-SINBV-luc vector in order to demonstrate the requirement for the viral enzymatic proteins for high levels of reporter gene expression. This is accomplished by digestion of pVGELVIS-SINBV-luc DNA with Bsp EI, purification with GENECLEAN, and ligation under dilute conditions. This construction is deleted of nonstructural gene sequences between bases 422-7,054 and is analogous to the pVGSP6GENd1Bsp construction described in Example 3, section C above and shown sche-

matically in FIG. 5. The construction described here is known as pVGELVIS-SINBVdIBsp-luc (abbreviated as dINSP ELVIS-luc). To link the luciferase gene directly to the MoMuLV LTR, the reporter is first inserted into the pCDNA3 vector (Invitrogen, San Diego, Calif.) between the Bam HI and Hind III sites. The luciferase fragment is derived from pGL2 plasmid exactly as described in Example 3 section B, above, and inserted into the 5428× bp fragment of pCDNA3 prepared by digestion with Hind III and Bam HI, treatment with CIAP, and purification on a 1% agarose/TBE gel. This construction is known as pCDNA3-luc. The U3 region of the MoMuLV LTR is amplified from the BAG vector using the PCR primers shown below as described in Example 2.

Forward primer: BAGBg12FI (buffer sequence/Bgl II recognition sequence/Mo-MLV LTR nts 1–22)

5'-TATATAGATCrAATGAAAGACCCACCTGTAGG (SEQ. ID NO. 15)

Reverse primer: BAGwt441R2 (SIN nts 5-1/Mo-MLV LTR nts 441–406):

5'-TCAATCCCCGAGTGAGGGGTTGTGGGCTCTT-TTATTGAGC (SEQ. ID NO. 16)

The amplification products are purified with GENECLEAN and the ends are first blunted with T4 DNA polymerase, then digested with Bgl II, purified with GENECLEAN™ and ligated into the pCDNA3-luc plasmid prepared by digestion with Hind III, blunting with the Klenow enzyme and 50 uM dNTPs, digestion with Bgl II, and purification by 1% agarose/TBE gel electrophoresis. This construction is known as LTR-luc.

The plasmids ELVIS-luc, dINSP ELVIS-luc, LTR-luc, and ELVIS-luc dlpro are each complexed with 10 ul of LIPOFECTAMINE™ and transfected into 5×10<sup>5</sup> BHK-21 cells contained in 35 mM petri plates. The luciferase activity is determined from each of three samples at 48 hrs. post-transfection. The results of this study, given in FIG. 10, demonstrate that the level of heterologous gene expression enhancement provided by the ELVIS system, compared to the same promoter linked directly to the heterologous gene is at least 10-fold. The comparatively low level of luciferase expression in cells transfected with the dINSP ELVIS-luc construction demonstrates that the expression enhancement is a direct result of functional Sindbis NSPs. The autocatalytic amplification of the reporter gene mRNA as depicted in FIG. 8 provides a significant advantage in terms of levels of gene expression, compared to primary transcription from simple promoter-heterologous gene constructions. Thus, as shown schematically in FIG. 8, after transfection of the ELVIS vector primary transcription in the nucleus and transport of the vector RNA to the cytoplasm leads to the synthesis of Sindbis NSPs which catalyze the expansion of heterologous gene mRNA via an antigenome intermediate which in turn serves as the template for production of genomic and subgenomic mRNA.

An experiment is performed to demonstrate the expression and rescue of RNA- and plasmid DNA (ELVIS)-based Sindbis expression vectors. For the RNA vectors, 5×10<sup>5</sup> BHK-21 cells contained in 35 mM petri plates are transfected with SIN-luc RNA, or co-transfected with SIN-luc RNA and SINDIBspEI RNA, complexed with LIPOFECTAMINE™. For the ELVIS vectors, 5×10<sup>5</sup> BHK-21 cells contained in 35 mM petri plates are transfected with ELVIS-luc, or co-transfected with ELVIS-luc and pVGELVISdIBspEI, whose construction is described in Example 7, complexed with LIPOFECTAMINE™. The results of this study, shown in FIG. 23 demonstrate clearly that the level of expression after transfection and transduction is similar between BHK

cells co-transfected with RNA or ELVIS vectors. Thus, the ELVIS vectors are used not only as plasmid DNA expression vectors, but additionally expression and helper vector ELVIS constructs can be cotransfected into cells to generate recombinant vector particles.

#### F. CONSTRUCTION OF MODIFIED DNA-BASED ALPHAVIRUS EXPRESSION VECTORS

The overall efficiency of the ELVIS vector, as determined by level of heterologous gene expression, is enhanced by several modifications to the pVGELVIS-SINBV-luc vector. These modifications include alternate RNA polymerase II promoters and transcription termination signals, the addition of intron sequences and ribozyme processing signals in the vector construct, and substitution with a smaller plasmid vector backbone. The construction of these modified ELVIS vectors is detailed below.

The modified ELVIS vector is assembled on the plasmid vector pBGS131 (ATCC #37443) which is a kanamycin resistant analogue of pUC 9 (Spratt et al., *Gene* 41:337–342, 1986). Propagation of pBGS131 is in LB medium with 10 ug/ml kanamycin.

The transcription termination signals from the SV40 early region or Bovine growth hormone are inserted between the Sac I and Eco RI sites of pBGS131. The SV40 nts between viral nts 2643 to 2563 containing the early region transcription termination sequences are isolated by PCR amplification using the primer pair shown below and the pBR322/SV40 plasmid (ATCC #45019) as template.

Forward primer SSVTT 2643 (buffer sequence/Sac I site/SV40 nts 2643–2613)

5'-TATATATGAGCTCTACAAATAAAGCAATAGC-ATCACAAATTTC (SEQ. ID NO. 35)

Reverse primer RSVTT2563R (buffer sequence/Eco RI site/SV40 nts 2563–2588):

5'-TATATGAATTCGTTTGGACAAACCACAACCT-AGAATG (SEQ. ID NO. 36)

The primers shown above are used in a PCR reaction with a three temperature cycling program as described throughout this example, using a 30 second extension period. The amplification products are purified with GENECLEAN™, digested with Sac I and Eco RI, purified again with GENECLEAN™, and the 90 bp fragment is ligated into the 3,655 bp fragment of pBGS131 resulting from digestion with Sac I and Eco RI, and treatment with CIAP. This construction is known as pBGS131-3'SV40TT

The Bovine growth hormone transcription termination sequences are isolated by PCR amplification using the primer pair shown below and the pCDNA3 plasmid (Invitrogen) as template.

Forward primer BGHTTF (buffer sequence/Sac I site/pCDNA3 nts 1132–1161):

5'-TATATATGAGCTCTAATAAAATGAGGAAATTG-CATCGCATGTGC (SEQ. ID NO. 37)

Reverse primer BGHTTR (buffer sequence/Eco RI site/pCDNA3 nts 1180–1154):

5'-TATATGAATTCATAGAATGACACCTACFCAG-ACAATGCGATGC (SEQ. ID NO. 38)

The primers shown above are used in a PCR reaction with a three temperature cycling program, using a 30 sec. extension period. The amplification products are purified with GENECLEAN™, digested with Sac I and Eco RI, purified again with GENECLEAN™, and the 58 bp fragment is ligated into the 3,655 bp fragment of pBGS131 resulting from digestion with Sac I and Eco RI, and treatment with CIAP. This construction is known as pBGS131-3'BGHTT.

In additional modifications to the ELVIS vector, the transcription termination sequences are fused directly to the

3'-end Sindbis sequences, resulting in deletion of the polyadenylate tract; or alternatively the antigenomic ribozyme sequence of hepatitis delta virus (HDV) is inserted between the 3'-polyadenylate tract of the ELVIS vector and the transcription termination signals.

The HDV ribozyme-containing construct is generated with PCR techniques and overlapping oligonucleotide primers which contain the minimal 84 nucleotide antigenomic ribozyme sequence (Perotta and Been, *Nature* 350:434-6, 1991). In addition to the HDV sequence, the primers contain flanking Sac I recognition sites for insertion at the 3' end of the ELVIS vector. The HDV ribozyme sequence is generated with the three overlapping primers shown below.

Forward primer SHDV1F (Buffer sequence/Sac I site/HDV RBZ seq.):

5'-TATATGAGCTCGGGTCGGCATGGCATCTCCA-CCTCCTCGCGGTCCG (SEQ. ID NO. 39)

Nested primer HDV17-68:

5'-TCCACCTCTCGCGGTCCGACCTGGGCATCC-GAAGGAGGACGCAC GTCCACT-3' (SEQ. ID NO. 40)

Reverse primer SHDV84R (Buffer sequence/Sac I site/HDV RBZ seq.):

5'-TATATGAGCTCCTCCCTTAGCCATCCGAGTGG-ACGTGCGTCCTCCTTC (SEQ. ID NO. 41)

The primers shown above are used in a PCR reaction with a three temperature cycling program as described throughout this example, using a 30 sec. extension period. The amplification products are purified with GENECLEAN™, digested with Sac I, purified again with GENECLEAN™, and the 94 bp fragment subsequently is ligated into plasmid vectors pBGS131-3'SV40TT or pBGS131-3'BGHTT that are digested with SacI under limiting conditions that linearize (cut 1 of 2 sites) and are treated with CIAP. These constructions are known as pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT. Insertion of the HDV ribozyme in both the correct orientation and in the correct Sac I site is determined by sequencing. In addition, longer or shorter HDV ribozyme sequences, or any other catalytic ribozyme sequence, may be readily substituted given the disclosure provided herein.

In the second vector 3'-end configuration, the SV40 or BGH transcription termination signals are fused directly to the 3'-end of the ELVIS vector corresponding to Sindbis nt 11,700 and the polyadenylate tract is deleted. This construction is accomplished according to the steps outlined above in Example 3, sections A and B for the assembly of the pKSSINBV and pKSSINBV-luc vectors. However, in this application the vector 3'-end primer does not contain a 25 polyadenylate tract. The 3'-end of the vector is synthesized with the primer pair shown below:

Forward Primer: SIN 11664F: (buffer sequence/Not I site/SIN nts 11664-11698):

5'-TATATGCGGCCGCGCTTTATTAATCAACAAAA-TTTTGTTTTAA (SEQ. ID NO. 42)

Reverse Primer: SSIN11700R (buffer sequence/Sac I site/SIN nts 11700-11655):

5'-TATATGAGCTCGAAATGTTAAAAACAAAATT-TTGTG (SEQ. ID NO. 43)

The primers shown above are used in a PCR reaction with a three temperature cycling program as described throughout this example, using a 30 sec. extension period. Assembly of the pKSSINBV and pKSSINBV-luc vectors is precisely as shown in Example 3, sections A and B. These constructions are known as pKSSINBVdIA and pKSSINBVdIA-luc.

The ELVIS expression vectors are assembled further onto the various 3' end processing plasmid constructions

described above. The Sindbis vectors containing a polyadenylate tract are combined with the plasmid constructions containing the HDV ribozyme sequence and the SV40 or BGH transcription termination signals. This construction corresponds to the insertion of pKSSINBV and pKSSINBV-luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids. Alternatively, the Sindbis vectors terminating precisely at the viral 3'end corresponding to viral nt 11,700 are linked directly to the SV40 or BGH transcription termination signals. This construction corresponds to the insertion of pKSSINBVdIA and pKSSINBVdIA-luc vector sequences into the pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids.

The Sindbis vectors pKSSINBV and pKSSINBV-luc are digested with Sac I and Bgl II, and the 5,522 bp (pKSSINBV) or 8211 bp (pKSSINBV-luc) fragments are purified by 1% agarose/TBE gel electrophoresis and inserted into the linearized pBGS131/HDV/3'SV40TT and pBGS131/HDV/3'BGHTT plasmids prepared by digestion with Sac I and Bgl II and treatment with CIAP. These constructions are known as:

pBGS131/dlproSINBV-luc/HDV/3'SV40TT

pBGS131/dlproSINBV-luc/HDV/3'BGHTT

pBGS131/dlproSINBV/HDV/3'SV40TT

pBGS131/dlproSINBV/HDV/3'BGHTT

Using the same strategy described above, the Sindbis vectors pKSSINBVdIA and pKSSINBVdIA-luc are digested with Sac I and Bgl II, and the 5,497 bp (pKSSINBVdIA) or 8186 bp (pKSSINBVdIA-luc) fragments are purified by 1% agarose/TBE gel electrophoresis and inserted into the linearized pBGS131/3'SV40TT and pBGS131/3'BGHTT plasmids prepared by digestion with Sac I and Bgl II and treatment with CIAP. These constructions are known as:

pBGS131/dlproSINBV-luc/3'SV40TT

pBGS131/dlproSINBV-luc/3'BGHTT

pBGS131/dlproSINBV/3'SV40TT

pBGS131/dlproSINBV/3'BGHTT

The addition of an RNA polymerase II promoter and Sindbis nucleotides 1-2289 is the last step required to complete the construction of the modified ELVIS expression vectors of the eight constructions shown below:

pBGS131/dlproSINBV-luc/HDV/3'SV40TT

pBGS131/dlproSINBV-luc/HDV/3'BGHTT

pBGS131/dlproSINBV/HDV/3'SV40TT

pBGS131/dlproSINBV/HDV/3'BGHTT

pBGS131/dlproSINBV-luc/3'SV40TT

pBGS131/dlproSINBV-luc/3'BGHTT

pBGS131/dlproSINBV/3'SV40TT

pBGS131/dlproSINBV/3'BGHTT

These eight constructions contain a unique Bgl II restriction site, corresponding to Sindbis nt 2289. The RNA polymerase II promoter and Sindbis nucleotides 1-2289 are inserted into these constructions by the overlapping PCR technique described for the pVGELVIS construction in Example 2. In order to insert the RNA polymerase II promoter and the 2289 Sindbis nts, the eight constructions shown above are digested with Bgl II and treated with CIAP.

The U3 region of the long terminal repeat (LTR) from Moloney murine leukemia virus (Mo-MLV) is positioned at the 5' viral end such that the first transcribed nucleotide is a single G residue, which is capped in vivo, followed by the Sindbis 5' end. Amplification of the Mo-MLV LTR in the first primary PCR reaction is accomplished in a reaction containing the BAG vector (Price et al., *PNAS* 84:156-160, 1987) and the following primer pair:

Forward primer: BAGBgl2F1 (buffer sequence/Bgl II recognition sequence/Mo-MLV LTR nts 1-22):

5'-TATATAGATCTAATGAAAGACCCACCTGTAGG  
(SEQ. ID NO. 15)

Reverse primer: BAGwt441R2 (SIN nts 5-1/Mo-MLV LTR nts 441-406):

5'-TCAATCCCCGAGTGAGGGGTTGTGGGCTCT-  
VTATTGAGC (SEQ. ID NO. 16)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 30 second extension period.

Amplification of the Sindbis 5' end in the second primary PCR reaction is accomplished in a reaction containing the pVGSP6GENrep clone and the following primer pair:  
Forward primer: (Mo-MLV LTR nts 421-441/SIN nts 1-16):

5'-CCACAACCCCTCACTCGGGGATTGACGGCG-  
TAGTAC (SEQ. ID NO. 17)

Reverse primer: (SIN nts 3182-3160):

5'-CTGGCAACCGGTAAGTACGATAC (SEQ. ID NO.  
18)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period.

The 457 bp and 3202 bp products from the primary PCR reactions are purified with GENE CLEAN™, and used together in a PCR reaction with the following primer pair:  
Forward primer: BAGBgl2F1 (buffer sequence/Bgl II recognition sequence/Mo-MLV LTR nts 1-22):

5'-TATATAGATCTAATGAAAGACCCACCTGTAGG  
(SEQ. ID NO. 15)

Reverse primer: (SIN nts 2300-2278):

5'-GGTAACAAGATCTCGTGCCGTG (SEQ. ID NO.  
19)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period. The 25 3'-terminal bases of the first primary PCR amplicon product overlap with the 25 5'-terminal bases of the second primary PCR amplicon product; the resultant 2,752 bp overlapping secondary PCR amplicon product is purified by 1% agarose/TBE electrophoresis, digested with Bgl II, and the 2,734 bp product is ligated into the eight ELVIS constructions described above. These constructions are named as shown below:

MpLTRELVIS-luc/D/S  
MpLTRELVIS-luc/D/B  
MpLTRELVIS/D/S  
MpLTRELVIS/D/B  
MpLTRELVIS-luc/S  
MpLTRELVIS-luc/B  
MpLTRELVIS/S  
MpLTRELVIS/B

Using the same overlapping PCR approach, the CMV promoter is positioned at the 5' viral end such that transcription initiation results in the addition of a single non-viral nucleotide at the Sindbis 5' end. Amplification of the CMV promoter in the first primary PCR reaction is accomplished in a reaction containing the pCDNA3 plasmid and the following primer pair:

Forward primer: pCBgl233F (buffer sequence/Bgl II recognition sequence/CMV promoter nts 1-22):

5'-TATATATAGATCTTTGACATTGATTATGACTAG  
(SEQ. ID NO. 44)

Reverse primer: SNCMV1142R (SIN nts 8-1/CMV pro nts 1142-1108):

5'-CCGTCAATACGGTTCACTAAACGAGCTCTGC-  
TTATATAGACC (SEQ. ID NO. 45)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 1 minute extension period.

Amplification of the Sindbis 5' end in the second primary PCR reaction is accomplished in a reaction containing the pVGSP6GENrep clone and the following primer pair:

Forward primer: CMVSIN1F (CMV pro nts 1124-1142/SIN nts 1-20):

5'-GCTCGTTTAGTGAACCGTATTGACGGCGTAGT-  
ACACAC (SEQ. ID NO. 46)

Reverse primer: (SIN nts 3182-3160):

5'-CTGGCAACCGGTAAGTACGATAC (SEQ. ID NO.  
18)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period.

The 600 bp and 3200 bp products from the primary PCR reactions are purified with GENE CLEAN™, and used together in a PCR reaction with the following primer pair:

Forward primer: pCBgl233F (buffer sequence/Bgl II recognition sequence/CMV promoter nts 1-22):

5'-TATATATAGATCTTTGACATTGATTATGACTAG  
(SEQ. ID NO. 44)

Reverse primer: (SIN nts 2300-2278):

5'-GGTAACAAGATCTCGTGCCGTG (SEQ. ID NO.  
19)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period.

The 26 3' terminal bases of the first primary PCR amplicon product overlaps with the 26 5' terminal bases of the second primary PCR amplicon product; the resultant 2,875 bp overlapping secondary PCR amplicon product is purified by 1% agarose/TBE electrophoresis, digested with Bgl II, and ligated into the four ELVIS constructions described above. These constructions are named as shown below:

MpCMVELVIS-luc/D/S  
MpCMVELVIS-luc/D/B  
MpCMVELVIS/D/S  
MpCMVELVIS/D/B  
MpCMVELVIS-luc/S  
MpCMVELVIS-luc/B  
MpCMVELVIS/S  
MpCMVELVIS/B

Using the same overlapping PCR approach, the SV40 early region promoter is positioned at the 5' viral end such that the major cap site of transcription initiation results in the addition of a single non-viral nucleotide at the Sindbis 5' end. Amplification of the SV40 promoter in the first primary PCR reaction is accomplished in a reaction containing the pBR322/SV40 plasmid (ATCC #45019) and the following primer pair:

Forward primer: B2SVpr250F (buffer sequence/Bgl II recognition sequence/SV40 nts 250-231):

5'-TATATATAGATCTGGTGTGGAAAGTCCCCAGGC  
(SEQ. ID NO. 47)

Reverse primer: SINSV5235R (SIN nts 13-1/SV40 nts 5235-10):

5'-CTACGCCGTCAATGCCGAGGCGGCCTCGGCC  
(SEQ. ID NO. 48)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 30 second extension period.

Amplification of the Sindbis 5' end in the second primary PCR reaction is accomplished in a reaction containing the pVGSP6GENrep clone and the following primer pair:

Forward primer: SVSIN1F (SV40 nts 3-5235/SIN nts 1-25):

5'-GGCCGCCTCGGCATTGACGGCGTAGTACACA-CTATTG (SEQ. ID NO. 49)

Reverse primer: (SIN nts 3182-3160):

5'-CTGGCAACCGGTAAGTACGATAC (SEQ. ID NO. 18)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period.

The 280 bp and 3,194 bp products from the primary PCR reactions are purified with GENECLEAN™, and used together in a PCR reaction with the following primer pair: Forward primer: B2SVpr250F (buffer sequence/Bgl II recognition sequence/SV40 nts 250-231):

5'-TATATATAGATCTGGTGTGGAAAGTCCCCAGGC (SEQ. ID NO. 47)

Reverse primer: (SIN nts 2300-2278):

5'-GGTAACAAGATCTCGTGCCGTG (SEQ. ID NO. 19)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 3 minute extension period.

The 25 3' terminal bases of the first primary PCR amplicon product overlaps with the 25 5' terminal bases of the second primary PCR amplicon product; the resultant 2,543 bp overlapping secondary PCR amplicon product is purified by 1% agarose/TBE electrophoresis, digested with Bgl II, and ligated into the four ELVIS constructions described above. These constructions are named as shown below:

MpSV40ELVIS-luc/D/S

MpSV40ELVIS-luc/D/B

MpSV40ELVIS/D/S

MpSV40ELVIS/D/B

MpSV40ELVIS-luc/S

MpSV40ELVIS-luc/B

MpSV40ELVIS/S

MpSV40ELVIS/B

The luciferase expression levels, after transfection of BHK-21 cells, are determined with each of the reporter gene containing complete modified ELVIS constructions detailed above, in order to determine the optimal desired configuration. The heterologous gene is inserted into the multiple cloning site of the ELVIS vector, as described for the insertion of the luciferase gene in Example 3, section B.

In order to increase the efficiency of the ELVIS system, in terms of functional vector RNA transported to the cytoplasm per nuclear DNA template, the SV40 small t antigen intron can be inserted into the ELVIS expression vectors. Insertion of the SV40 small t antigen intron sequences into the Xho I site immediately downstream of the 5' Sindbis sequences is accomplished by limited digestion (cut 1 of 2 sites); or, alternatively at the unique Not I site immediately upstream of the 3' Sindbis sequences.

For insertion into the Xho I site of the ELVIS vectors, amplification of the SV40 small t antigen intron sequences is accomplished in a reaction containing the pBR322/SV40 plasmid (ATCC #45019) and the following primer pair: Forward primer: XSVSD4647F (buffer sequence/Xho I recognition sequence/SV40 nts 4647-4675):

5'-TATATATCTCGAGAAGCCTCAAGGTAAATAT-AAAATTIACC (SEQ. ID NO. 50)

Reverse primer: XSVSA4562R (buffer sequence/Xho I recognition sequence/SV40 nts 4562-4537):

5'-TATATATCTCGAGAGGTTGGAATCTAAAA-TACACAAAC (SEQ. ID NO. 51)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 30 second extension period. The amplification products are purified with GENECLEAN™, digested with Xho I, re-purified with GENECLEAN™ and inserted into Xho I linearized (by limited digest) and CIAP treated complete modified ELVIS vectors described above. Insertion of the SV40 small t antigen intron in the correct orientation in the ELVIS vector is determined by sequencing.

For insertion into the Not I site of the ELVIS vectors, amplification of the SV40 small t antigen intron sequences is accomplished in a reaction containing the pBR322/SV40 plasmid and the following primer pair:

Forward primer: NSVSD4647F (buffer sequence/Not I recognition sequence/SV40 nts 4647-4675):

5'-TATATATGCGGCCGCAAGCTCTAAGGTAAAT-ATAAAATTIACC (SEQ. ID NO. 52)

Reverse primer: XSVSA4562R (buffer sequence/Not I recognition sequence/SV40 nts 4562-4537):

5'-TATATATGCGGCCGCGAGGTTGGAATCTAAAA-TACACAAAC (SEQ. ID NO. 53)

The primers shown above are used in a PCR reaction with a three temperature cycling program using a 30 second extension period. The amplification products are purified with GENECLEAN™, digested with Not I, re-purified with GENECLEAN™ and inserted into Not I linearized and CIAP treated complete modified ELVIS vectors described above. Insertion of the SV40 small t antigen intron in the correct orientation in the ELVIS vector is determined by sequencing. Alternatively, the SV40 small t antigen may be inserted at other sites within the ELVIS vector, which do not impair function of the vector, using the disclosure provided herein.

The luciferase expression levels, after transfection of BHK-21 cells with the SV40 small t antigen intron containing ELVIS vectors, are assayed in order to determine the optimal desired configuration. The heterologous gene is inserted into the multiple cloning site of the ELVIS vector, as described for the insertion of the luciferase gene in Example 3, section B.

A linker sequence is inserted into the pKSSINBV and into the pVGELVIS-SINBV constructs to facilitate the insertion of heterologous sequences. The linker is constructed using two complementary 35 nt oligonucleotides that form a duplex with Xho I and Xba I compatible sticky ends when hybridized.

SINBVLinkF: 5'TCGAGCACGTGGCGCGCCTGAT-CACGCGTAGGCCT (SEQ. ID NO. 54)

SINBVLinkR: 5'CTAGAGGCCTACGCGTGATCAG-GCGGCCACGTGC (SEQ. ID NO. 55)

The oligonucleotides are phosphorylated with T4 polynucleotide kinase, heated to 90° C., and slow cooled to allow hybridization to occur. The hybrid is then ligated to the 10.6 kb fragment of pKSSINBV-Luc obtained after digestion with XhoI and XbaI, followed by treatment with alkaline phosphatase and agarose gel purification. The resulting construct contains Xho I, Pml I, Asc I, Bcl I, Mlu I, Stu I, Xba I, and Not I as unique sites between the Sindbis junction region and the Sindbis 3' end. This construct is known as pKSSINBV-Linker.

This linker also is cloned into the pVGELVIS-SINBV constructs. The linker is inserted by digestion of pVGELVIS-SINBV-luc with Sfi I and Not I. The 10.1 kb fragment is agarose gel purified, and this fragment was ligated to the gel purified 2.6 kb fragment from a Sfi I/Not I digest of pKSSINBV-Linker. The resulting construct contains Xho I, Pml I, Asc I, Mlu I, and Not I as unique sites



between the Sindbis junction region and the Sindbis 3' end. This construct is known as pVGELVIS-SINBV-Linker.

Additional experiments are performed to compare the relative expression activities of Sindbis RNA and DNA reporter vectors in transfected BHK cells (FIG. 22). Luciferase expression is approximately 30-fold higher in cells transfected with in vitro transcribed SIN-luc RNA, compared to the level in cells transfected with ELVIS-luc plasmid DNA (FIG. 22A). The data also demonstrate that direct linkage between the Sindbis virus 3'-end and two different transcription termination/polyadenylation signals, resulting in deletion of the synthetic A<sub>25</sub> tract, decreased the activity of the DNA vector by more than three orders of magnitude (FIG. 22A). However, measurable expression of luciferase is detected, suggesting that these 3' end modified Sindbis DNA vectors do function in transfected cells at some low level. Additionally, the insertion of a HDV ribozyme processing sequence, downstream of the A<sub>25</sub> tract, increases activity of the DNA vector 3-4 fold over the ELVIS-luc vector or an analogous construct with the HDV ribozyme inserted in a reverse orientation (FIG. 22B).

Based on the decreased expression levels observed when the synthetic A<sub>25</sub> tract is deleted, additional constructs related to MpELVIS/S and MpELVIS/B are then made exactly as outlined in the above example utilizing the Sindbis sequences from the pKSSINBV and pKSSINBV-luc vectors to include the A<sub>25</sub> tract. These constructions are named as shown below:

MpLTRELVIS-luc/A/S  
MpLTRELVIS-luc/A/B  
MpLTRELVIS/A/S  
MpLTRELVIS/A/B  
MpCMVELVIS-luc/A/S  
MpCMVELVIS-luc/A/B  
MpCMVELVIS/A/S  
MpCMVELVIS/A/B  
MpSV40ELVIS-luc/A/S  
MpSV40ELVIS-luc/A/B  
MpSV40ELVIS/A/S  
MpSV40ELVIS/A/B

#### G. REPORTER GENE EXPRESSION IN RODENTS INOCULATED INTRAMUSCULARLY WITH ELVIS VECTORS

Using techniques described above, the lacZ gene encoding the  $\beta$ -galactosidase reporter protein was cut from the plasmid pSV- $\beta$ -galactosidase (PROMEGA CORP, Madison, Wis.) and substituted into the ELVIS-luc plasmid DNA vector in place of luciferase. To examine in vivo gene expression from ELVIS vectors, Balb/c mice and rats are injected intramuscularly (i.m.) with ELVIS- $\beta$ -gal or ELVIS-luc plasmid DNA vectors. FIG. 24 demonstrates the in vivo expression of  $\beta$ -galactosidase in muscle tissue taken from a rat and stained with X-gal at three days post i.m. injection. Mice injected with ELVIS- $\beta$ -gal also demonstrate positively staining blue muscle fibers. Luciferase expression levels from muscle which were between 75- and 300-fold higher than control levels were detected in 3/4 Balb/c mice at two days post i.m. inoculation with ELVIS-luc plasmid. In other experiments, C3H/HeN mice were injected i.m. with ELVIS vectors expressing either the hepatitis B virus core (HBV-core) or hepatitis B virus e (HBV-e) proteins. Using ELISA detection systems, both HBV-core- and HBV-e-specific IgG antibodies were detected in serum samples collected from the mice 10 days following the second injection with the vectors. These experiments demonstrate that Sindbis-derived DNA vectors are able to express foreign genes in vivo, in rat and mouse muscle.

#### H. ADAPTATION OF ALPHAVIRUS EXPRESSION VECTORS

The following description details how to identify alphaviral vectors according to the invention adapted to grow in cells of a particular eukaryotic species. Specifically, adaptation of Sindbis virus variants adapted to grow in human cells is disclosed. As those in the art will appreciate, the following procedure can be employed to adapt other alphaviral vectors to particular eukaryotic species.

To adapt Sindbis viral vectors derived from BHK-21 cells to human cells, Sindbis viral vectors produced in accordance with this invention are propagated by serial passage in HT1080 (ATCC acc. no. CCL 121) and DM150 (a human cell line established from a primary melanoma tumor) cell lines in order to select variants which are able to establish high titer productive infections in human cells. Isolation of Sindbis variants adapted to human cells is accomplished by the following method: HT1080 and DM150 cells propagated in DMEM with 10% fetal calf serum (FCS) are infected at a multiplicity of infection of 5 with the virus contained in a small volume to facilitate infection. At one hour post infection, the inoculum is removed, the monolayer washed several times with DMEM, and the media replenished. The viral supernatant is harvested at 7 hours post infection, clarified by centrifugation, and divided into three aliquots. Two aliquots are frozen and the other aliquot is split and used to infect fresh HT1080 and DM150 monolayers. This process is repeated at least 10 times or as sufficient to generate variants which replicate efficiently in human cells. After each serial passage, plaque assays are performed in BHK cells or the homologous cell line in which the virus was propagated to determine an increase in virus titer in human cell lines. Sindbis variants adapted to human cells which contain the highest level of virus produced during serial HT1080 or DM150 cell line passage are then isolated from supernatants by three rounds of plaque purification. The phenotype of the plaque purified human variant is verified by determining its growth properties in human cell lines.

In an alternative approach, variants which are able to establish high titer productive infections in human cells are isolated by plaque morphology. Human cell lines, for example HT1080 and DM150, are infected at low multiplicity of infection with Sindbis virus grown in BHK-21 cells and overlaid with agar. At 24-30 hours post infection, large plaques, indicative of variants able to propagate efficiently in human cells, are picked. The variants are then purified by two additional serial rounds of plaque purification. The phenotype of candidate Sindbis variants can then be determined by comparing growth properties on human and BHK-21 cells with BHK-21 cell-propagated Sindbis virus.

Another similar approach enables the production of Sindbis variants which establish high titer persistent, i.e., noncytotoxic, infection of human cells. Specifically, human cells are infected with a Sindbis virus preparation containing a high percentage of defective interfering (DI) particles isolated by undiluted serial passage in HT1080 or DM150 cells. Cells which survive infection with this DI contaminated Sindbis stock are allowed to proliferate. Virus is isolated from the supernatant and purified by multiple rounds of plaque purification in BHK-21 or human cells. The desired phenotype of the Sindbis variant is verified by determining its ability to establish persistent noncytotoxic persistent infection in human cell lines.

Following identification of one or more Sindbis variants having the desired phenotype, purified viral RNA from the

Sindbis variant is cloned and characterized in order to identify the nonstructural and structural genes and noncoding region changes which contribute to the observed phenotype. Sindbis variant genomic cDNA cloning is accomplished by RT-PCR, as detailed in Example 1 and the phenotype of the molecularly cloned virus strains is verified.

Viral genetic determinants can be mapped by identifying at what level Sindbis infection of human cells is inhibited, i.e., at the stage of adsorption, entry, replication, or assembly. The 5'-end, junction region, and nonstructural and noncoding region genetic determinants responsible for human variant phenotypes can be mapped by exchanging defined regions from pKSSINBV-luc, supra, with corresponding regions from the variant cDNA to produce various "test" SIN-luc vectors. After packaging by co-transfection, the level of luciferase expression in DM150, HT1080, and BHK cells infected with either pKSSINBV-luc or the "test" SIN-luc vector is compared. Exchanging defined regions between vectors may be accomplished by exploiting convenient restriction endonuclease recognition sites, for example (Viagene SIN-BV numbering): Afl II (4573), Age I (3172, 6922), Avr II (4281), Bgl II (2289), Bpu 1102I (5602, 6266), BsaBI (2479) BstBI (4706, 6450), Eco47III (1407), Hpa I (6920), Mun I (42, 2785), Nru I (2324), Nsi I (2006, 6462), PflMI (4374), Sfi I (5122), and XhoI (7645). Precise nucleotide identification of genetic determinants resulting in the human variant phenotype can be accomplished by sequencing.

The 3'-end nonstructural and coding region genetic determinants responsible for the variant phenotype may be mapped by exchanging defined regions with the dl-BspEI cotransfection packaging vector. After packaging by co-transfection, the level of luciferase expression in DM150, HT1080, and BHK cells infected with pKSSINBV-luc packaged with the dl-BspEI cotransfection packaging vector or with the "test" dl-BspEI cotransfection packaging vector is compared. Exchanging defined regions between vectors may be accomplished by exploiting convenient restriction endonuclease recognition sites, for example (Viagene genomic Sindbis numbering): AatII (8000), Afl II (7969, 8836), Aval (9414), BclII (9356), Bpu11021I (8911), BsiWI (10379), BspMII (7054), Bsu36I (8892), EcoNI (10048, 10923), EcoRI (9077), KasI (10036, 11308), NruI (8329), PflMI (9554), PmlI (8070), SalI (9589, 11085), SmaI (9416), SphI (10379), StuI (8572), and (9414). Precise nucleotide identification of genetic determinants resulting in the human variant phenotype can be accomplished by sequencing.

#### I. RECOMBINANT PROTEIN EXPRESSION

The eukaryotic layered vector initiation systems of the invention can be used to direct the expression of one or more recombinant proteins in transformed or transfected eukaryotic host cells. A representative example of a recombinant protein which may be expressed using a eukaryotic layered vector initiation system is insulin.

The gene encoding human insulin was identified in 1980 by Bell, et al. [*Nature*, vol. 284, pp. 26-32]. The entire coding region for human preproinsulin (hpi) can be cloned from a variety of sources, e.g., a human pancreatic cDNA library [Clontech, Palo Alto, Calif., catalog no. HL1163a] using standard PCR techniques. Primers for amplifying the coding region flank the 5' and 3' ends of the gene. The 5' primer includes an XhoI site and the 3' primer includes a NotI recognition sequence. After PCR amplification, the reaction products are purified using GENECLEAN™, followed by XhoI and NotI digestion. The DNA is then gel purified and ligated into XhoI/NotI cleaved, CIAP-treated pVGLVIS-SINBV, infra, to make pELVS-hppi.

Alternatively, the hpi amplicon is inserted into XhoI/NotI cleaved, CIAP-treated SIN-BV, infra, to make pSIN-BV-hppi. RNA from Sac I-linearized pSIN-BV-hppi plasmid is synthesized in vitro as described in Example 3. Production of SIN-BV-hppi recombinant vector particles is accomplished by transfection of LIPOFECTIN™-complex SIN-BV-hppi RNA into the Sindbis vector packaging cell lines as described in Example 7. Generation of vector particles having expression vectors derived from Sindbis variants which establish high titer persistent noncytotoxic infection of human cells is accomplished by the same procedure.

pELVS-hppi is then introduced (e.g., by electroporation or by complexing with lipofectamine) into a suitable eukaryotic host cell, preferably an undifferentiated cell, for instance, F9 cells, infra. The transformed cells are then grown in the presence of G418 under suitable nutrient conditions (i.e., an appropriate medium, such as DMEM, including any required supplements, at 37° C.). The cells can be grown in a variety of formats, including in roller bottles, cell hotels, and bioreactors. Recombinant protein production is initiated by adding retinoic acid or another suitable inducing agent to the medium. At 12 to 48 hours post-vector induction, the optimal level of insulin is expressed into the medium and is recovered according to techniques known in the art. The insulin is recovered from the cell supernatants up to 18 hrs from the time in which the vector establishes a cytotoxic infection. Recovery of insulin from cells infected with expression vectors derived from Sindbis human cell variants may be harvested over a period extending to 3-5 days post induction. Insulin so produced is recovered according to techniques known in the art. The isolated recombinant protein may then be formulated in any of a number of pharmaceutical compositions suitable for human administration.

#### J. LYOPHILIZED EUKARYOTIC LAYERED VECTOR INITIATION SYSTEM VACCINES

One aspect of the invention concerns the use of eukaryotic layered vector initiation systems according to the invention as vaccines to immunize a human patient's or non-human animal's immune system against a particular disease. Such vaccines can be employed either prophylactically or therapeutically to prevent or treat disease. Diseases which may be treated with such vaccines include those caused by various pathogenic agents, such as procaryotic or eukaryotic microorganisms or viruses, or cancer.

For example, each of the vector constructs described herein and containing the heterologous sequence of a suitable antigen is readily lyophilized for long term stability. Upon re-hydration in an appropriate diluent, administration is performed and subsequent expression occurs. Additional alphavirus vector constructs not disclosed in the present invention, including those described in the literature (see Hahn et al., *Proc Natl Acad Sci USA* 89: 2679-2683, 1992) are readily convertible to a eukaryotic layered vector initiation system format by those skilled in the art and using the knowledge provided herein. Conversion of transient alphavirus vector systems to the format of a eukaryotic layered vector initiation system thus modify the duration of heterologous sequence expression to that of a more permanent and stable expression system. Advantages of this permanent and stable system include longer term expression, allowing greater prophylactic and therapeutic effects in both medical and veterinary applications.

#### K. EUKARYOTIC LAYERED VECTOR INITIATION SYSTEMS FOR PLANT APPLICATIONS

Given the disclosures provided herein, the adaptation of eukaryotic vector initiation system technologies to plant

application is readily performed by those skilled in the art. For illustration purposes, any of several positive-stranded plant viruses (for example, potato virus X (PVX, Huisman et al., *J. Gen. Virol.* 69:1789-1798, 1988), tobacco mosaic virus (TMV, Goelet et al., *Proc. Natl. Acad. Sci. USA* 79:5818-5822, 1982), and tobacco etch virus (TEV, Allison et al., *Virology* 154:9-20, 1986), see also, specifications) may be converted to a cDNA form using PCR and specific oligonucleotide primers, chosen from published sequences, as described in Example 1. After assembly of a full-length genomic clone linked to a bacteriophage RNA polymerase promoter, and determination of infectivity of in vitro synthesized transcripts, the cDNA is exchanged into a vector containing an RNA polymerase II promoter and transcription termination/polyadenylation sequence, as described in Example 2. For plant applications, such promoter and termination sequences are chosen from the appropriate plant systems (e.g., CaMV 35S promoter (Guilley et al., *Cell* 30:763-773, 1982), and nopaline synthase promoter and transcription termination sequence (Sanders et al., *Nucleic Acids Res.* 15:1543-1558). Vector constructs derived from these infectious genomic cDNA clones is subsequently accomplished using any of the approaches described in the present invention (e.g., use of subgenomic promoters, replacement of structural protein genes, use of IRES sequences). Specific applications of such plant eukaryotic layered vector initiation systems may include, but are not limited to, the expression of host-derived resistance sequences, pathogen-derived resistance sequences (e.g., protein-encoding, nonprotein-encoding, and defective interfering sequences), and growth promoting sequences, by the creation of transgenic plants harboring such systems.

#### L. TRANSGENIC ANIMAL APPLICATIONS

In accordance with the non-parenteral administration the present invention, the gene delivery vehicles, particularly those comprised of unencapsidated nucleic acid, may be complexed with a polycationic molecule to provide polycation-assisted non-parenteral administration. Such a method of gene delivery facilitates delivery of a gene via mediation by a physical particle comprised of multiple components that augment the efficiency and specificity of the gene transfer. In particular, polycationic molecules, such as polylysine and histone, have been shown to neutralize the negative charges on a nucleic acid molecule and to condense the molecule into a compact form. This form of molecule is transferred with high efficiency in cells, apparently through the endocytic pathway. The uptake in expression of the nucleic acid molecule in the host cell results after a series of steps, as follows: (1) attachment to cell surface; (2) cell entry via endocytosis or other mechanisms; (3) cytoplasmic compartment entry following endosome release; (4) nuclear transport; and (5) expression of the nucleic acid molecule carried by the gene delivery vehicle. In a further preferred embodiment, multi-layer technologies are applied to the polycation-nucleic acid molecule complex to facilitate completion of one or more of these steps. For example, a ligand such as asialoglycoprotein, transferrin, and immunoglobulin may be added to the complex to facilitate binding of the cell complex to the cell surface, an endosomal disruption component (e.g., a viral protein, a fusogenic peptide such as the n-terminus of the influenza virus hemagglutinin or an inactivated virus) is added to facilitate the release of DNA from the endosome, or a nuclear protein (or a peptide containing a nuclear localization signal) is added to facilitate the transport of the DNA into the nucleus. In a further preferred embodiment, the composition comprising the complex includes inactivated adenovirus particles

(Curiel, D. T., et al., *PNAS* 88: 8850-8854, 1991; Cristiano, R. J., *PNAS* 90: 2122-2126 1993; Cotten, M., et al., *PNAS* 89: 6094-6098 1992; Lozier, J. N., et al., *Human Gene Therapy* 5: 313-322, 1994; Curiel, D. T., et al., *Human Gene Therapy* 3: 147-154, 1992; Plank, C. et al., *Bioconjugate Chem.* 3: 533-539, 1992; Wagner, E. et al., *PNAS* 88: 4255-4259, 1991). The assorted components comprising the multi-layer complex may be varied as desired, so that the specificity of the complex for a given tissue, or the gene expressed from the gene delivery vehicle, may be varied to better suit a particular disease or condition.

As noted above, various methods may be utilized to administer gene delivery vehicles of the present invention, including nucleic acids which encode the immunogenic portion(s) discussed above, to warm-blooded animals such as humans, directly. Suitable methods include, for example, various physical methods such as direct DNA injection (Acsadi et al., *Nature* 352:815-818, 1991), and microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991).

Within an in vivo context, the gene delivery vehicle can be injected into the interstitial space of tissues including muscle, brain, liver, skin, spleen or blood (see, WO 90/11092). Administration may also be accomplished by intravenous injection or direct catheter infusion into the cavities of the body (see, WO 93/00051), discussed in more detail below.

It is generally preferred that administration of the gene delivery vehicles at multiple sites be via at least two injections. In this regard, suitable modes of administration include intramuscular, intradermal and subcutaneous injections, with at least one of the injections preferably being intramuscular. In particularly preferred embodiments, two or more of the injections are intramuscular. However, although administration via injections is preferred, it will be evident that the gene delivery vehicles may be administered through multiple topical or separate ocular administrations. Further, a number of additional routes are suitable for use within the present invention when combined with one or more of the routes briefly noted above, including intraperitoneal, intracranial, oral, rectal, nasal, vaginal and sublingual administration. Methods of formulating and administering the gene delivery vehicles at multiple sites through such routes would be evident to those skilled in the art and are described in U.S. Ser. No. 08/366,788 and U.S. Ser. No. 08/367,071 incorporated herein by reference in their entirety.

#### M. VETERINARY APPLICATIONS

From the description provided herein, those skilled in the art will appreciate that the alphavirus vector constructs, recombinant alphavirus particles, and eukaryotic layered vector initiation systems provided by the present invention can also be readily utilized in non-human animal (e.g., veterinary) applications. Such applications may include prophylactics (e.g., vaccines), immunotherapeutics, and palliatives. Within such aspects, compositions and methods are provided for administering an alphavirus vector construct, recombinant alphavirus particle, or eukaryotic layered vector initiation system which is capable of preventing, inhibiting, stabilizing or reversing infectious diseases in non-human animals.

Specifically, within one aspect of the present invention, compositions and methods are provided for stimulating an immune response (either humoral or cell-mediated) to a pathogenic agent, such that the pathogenic agent is either killed or inhibited. Representative examples of pathogenic agents of veterinary importance include bacteria, fungi, parasites and viruses.

More specifically, sequences which encode immunoreactive polypeptides of the pathogenic agents may, in certain embodiments, be chosen from a group that includes the Bunyaviridae (e.g., Rift Valley Fever virus (Giorgi et al., *Virology* 180:738-753, 1991; Collett et al., *Virology* 144:228-245, 1985)), Paramyxoviridae (e.g., Newcastle disease virus (Millar et al., *J. Gen. Virol.* 69:613-620, 1988; Chambers et al., *Nucl. Acid. Res.* 14:9051-9061, 1986; Schaper et al., *Virology* 165:291-295, 1988), and canine distemper virus (Curran et al., *J. Gen. Virol.* 72:443-447, 1991; Barrett et al., *Virus Res.* 8:373-386, 1987; Bellini et al., *J. Virol.* 58:408-416, 1986)), Togaviridae (e.g., WEE virus (Weaver et al., *Virology* 197:375-390, 1993), EEE virus (Chang et al., *J. Gen. Virol.* 68:2129-2142, 1987), and VEE virus (Kinney et al., *Virology* 152:400-413, 1986)), Rhabdoviridae (e.g., vesicular stomatitis virus (Gill et al., *Virology* 150:308-312, 1986; Gallione et al., *J. Virol.* 46:162-169, 1983; Banerjee et al., *Virology* 137:432-438, 1984), and rabies virus (Tordo et al., *Nucl. Acid. Res.* 14:2671-2683, 1986; Hiramatsu et al., *Virus Genes* 7:83-88, 1993; Kieny et al., *Nature* 312:163-166, 1984)), Coronaviridae (e.g., transmissible gastroenteritis virus (Britton et al., *Molec. Micro.* 2:89-99, 1988; Godet et al., *Virology* 188:166-175, 1992; Jackwood et al., *Adv. Exp. Med. and Biol.* 342:43-48, 1993), and feline infectious peritonitis virus (Reed et al., *Adv. Exp. Med. and Biol.* 342:17-21, 1993)), Reoviridae (e.g., porcine rotavirus (Burke et al., *J. Gen. Virol.* 75:2205-2212, 1994; Nishikawa et al., *Nucl. Acid. Res.* 16:11847, 1988)), Orthomyxoviridae (e.g. equine influenza (Gibson et al., *Virus Res.* 22:93-106, 1992; Dale et al., *Virology* 155:460-468, 1986)), Picomaviridae (e.g., FMD virus (Graham et al., *Virology* 176:524-530, 1990; Brown et al., *Gene* 75:225-233, 1989; Fross et al., *Nucl. Acid. Res.* 12:6587-6601, 1984)), and Herpesviridae (e.g., equine herpesvirus (Crabb et al., *J. Gen. Virol.* 72:2075-2082)).

In other embodiments, the sequences which encode immunoreactive polypeptides of the pathogenic agents may be chosen from a group that includes the agents of coccidiosis (e.g., *Eimeria* *Acerulina*, *E. tenella*, *E. maxima* (Talebi et al., *Infect. Immun.* 62:4202-4207, 1994; Pasamotites et al., *Mol. Biochem. Parasit.* 57:171-174, 1993; Tomley et al., *Mol. Biochem. Parasit.* 49:277-288, 1991; Castle et al., *J. of Parasit.* 77:384-390, 1991; Jenkins et al., *Exp. Parasit.* 70:353-362, 1990)), anaplasmosis (e.g., *Anaplasma marginale* (McGuire et al., *Vaccine* 12:465-471, 1994; Palmer et al., *Infect. Immun.* 62:3808-3816, 1994; Oberle et al., *Gene* 136:291-294, 1993; Barbet et al., *Infect. Immun.* 59:971-976, 1991; Barbet et al., *Infect. Immun.* 55:2428-2435, 1987)), babesiosis (e.g., *Babesia bovis* (Suarez et al., *Infect. Immun.* 61:3511-3517, 1993; Hines et al., *Mol. Biochem. Parasit.* 55:85-94, 1992; Jamer et al., *Mol. Biochem. Parasit.* 55:75-83, 1992; Suarez et al., *Mol. Biochem. Parasit.* 46:45-52, 1991)), theileriosis (e.g. *Theileria parva* (Nene et al., *Mol. Biochem. Parasit.* 51:17-27, 1992; Iams et al., *Mol. Biochem. Parasit.* 39:47-60, 1990)), malaria (e.g. *Plasmodium falciparum* (Haeseleer et al., *Mol. Biochem. Parasit.* 57:117-126, 1993)), salmonellosis (*Salmonella typhimurium* and *S. dublin*), bovine and ovine mastitis (*Staphylococcus aureus*), bovine tuberculosis (*Mycobacterium bovis*), pseudotuberculosis (*Yersinia pseudotuberculosis*), coccidioidomycosis (*Coccidioides immitis*), cryptococcosis (*Cryptococcus neoformans*), anthrax (*Bacillus anthracis*), brucellosis (*Brucella abortus* and *B. suis*), and leptospirosis (*Leptospira interrogans* and *L. biflexa*).

To illustrate this aspect in more detail, methods used in constructing recombinant alphavirus vectors and eukaryotic

layered vector initiation systems containing these sequences for veterinary application are described for two of the above pathogenic agents (one viral and one parasitic). The construction of additional alphavirus vectors and eukaryotic layered vector initiation systems is readily accomplished by those skilled in the art, based on the following methodologies and using sequences from other related or non-related pathogenic agents. In the case of foot-and-mouth disease virus (FMDV), a cassette comprising each of the four P1 capsid proteins (1A, 1B, 1C, 1D) and the 3C protease responsible for their post-translational cleavage is obtained as plasmids MR1 or MR2 from Graham et al. (*Virology* 176:524-530, 1990). Plasmid MR1 or MR2 is digested with the enzymes HindIII and DraI to remove the FMDV P1 cassette, followed by fill-in of the HindIII terminus with Klenow, and purification from a 1% agarose gel using GENECLAN™. Plasmid vectors pKSSINBV and pVGELVIS-SINBV (see Example 3) are digested with XhoI and the termini also made blunt using Klenow, followed by treatment with CIAP and purification from a 1% agarose gel using GENECLAN™. The purified fragments are subsequently ligated to generate the alphavirus vector construct pKSSIN-FMDV and eukaryotic layered vector initiation system plasmid pVGELVIS-FMDV. The purified FMDV sequences are also readily inserted into any of the other vector constructs described in this invention (see Example 3). Packaging of the FMDV-containing alphavirus vector construct pKSSIN-FMDV can be accomplished as described in Example 7.

For construction of a recombinant alphavirus vector construct or eukaryotic layered vector initiation system comprising sequences from a pathogenic agent of anaplasmosis, the major surface protein 2 (MSP-2) of *A. marginale* is obtained by PCR amplification from plasmid pCKR11.2 (Palmer et al., *Infect. Immun.* 62:3808-3816, 1994) using the following oligonucleotide pair, each containing a flanking XhoI site:

forward primer (AM-MSP-2F):

5'-TATATCTCGAGACCACCATGAGTGCTGTAA-GTAATAGGAAGC (SEQ. ID NO. 115)

reverse primer (AM-MSP-2R):

5'-TATATCTCGAGCTAGAAGGCCAAACCTAACACCCCAAC (SEQ. ID NO. 116)

A standard three temperature cycling protocol is performed as described previously using THERMALASE™ thermostable polymerase, the oligonucleotide pair, and plasmid pCKR11.2 as template. Following amplification, the MSP-2 amplicon is purified using GENECLAN™, digested with XhoI, and re-purified with GENECLAN™. Plasmid vectors pKSSINBV and pVGELVIS-SINBV (see Example 3) also are digested with XhoI, followed by treatment with CIAP and subsequent ligation to the MSP-2 fragment to generate the alphavirus vector construct pKSSIN-MSP2 and eukaryotic layered vector initiation system plasmid pVGELVIS-MSP2. The purified MSP-2 sequences are also readily inserted into any of the other vector constructs described elsewhere in this specification (e.g., Example 3). Packaging of the MSP-2-containing alphavirus vector construct pKSSIN-MSP2 can be accomplished as described in Example 7.

#### Example 4

##### A. INSERTION OF ADENOVIRUS EARLY REGION E3 GENE INTO SINDBIS VECTORS

In order to inhibit the host CTL response directed against viral specific proteins expressed in vector infected cells, in applications where repeated administration of the therapeutic

tic is desired, the Adenovirus type 2 (Ad 2) E3/19K gene ATCC No. VR-846 is cloned into the pKSSINdJRsirc plasmid, immediately downstream from the junction region core. Briefly, Ad 2 is propagated in a permissive cell line, for example HeLa or Vero cells, and after evidence of cytopathologic effects, virions are purified from the cell lysate, and the Ad 2 DNA is purified from the virus.

The Ad 2 DNA E3/19K gene, including the amino terminal signal sequence, followed by the intraluminal domain and carboxy terminal cytoplasmic tail which allows the E3 19K protein to embed itself in the endoplasmic reticulum, is located between viral nucleotides 28,812 and 29,288. Isolation of the Ad 2 E3 19K gene from the viral genomic DNA is accomplished by PCR amplification, with the primer pair shown below:

Ad 2 E3 Forward primer (Ad 2 nucleotides 28,812-28,835):

5'-TAT ATC TCC AGA TGA GGT ACA TGA TTT TAG GCT TG-3' (SEQ. ID NO. 56)

Ad 2 E3 Reverse primer (Ad 2 nucleotides 29,241-29,213):

5'-TAT ATA TCG ATT CAA GGC ATT TTC TTT TCA TCA ATA AAA C (SEQ ID NO.57)

In addition to the Ad 2 complementary sequences, both primers contain a five nucleotide 'buffer sequence' at their 5' ends for efficient enzyme digestion of the PCR amplicon products. This sequence in the forward primer is followed by the Xho I recognition site, and in the reverse primer this sequence is followed by the Cla I recognition site. Thus, in the 5' to 3' direction, the E3/19K gene is flanked by Xho I and Cla I recognition sites. Amplification of the E3/19K gene from Ad 2 DNA is accomplished with the following PCR cycle protocol:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.17	5
72	3.5	
94	0.5	30
70	3.5	
72	10	10

Following amplification, the 451 bp amplicon is purified on a 1.5% agarose gel, and digested with the Xho I and Cla I enzymes. pKSSINdJRsirc plasmid is partially digested with ClaI. Plasmid that has been digested only once is isolated by gel electrophoresis then digested with XhoI. The large fragment is isolated by gel electrophoresis and ligated to the digested PCR amplicon. This clone is designated pKSSINdJRsircAdE3. Using the same cloning strategy, the Ad 2 E3/19K gene may be inserted into any of the modified synthetic junction region vectors or ELVIS vectors described in Example 3.

#### B. INSERTION OF THE HUMAN CYTOMEGALOVIRUS H301 GENE INTO SINDBIS VECTORS

In order to inhibit the host CTL directed response against viral specific proteins expressed in vector infected cells in applications where repeated administration of the therapeutic is desired, the human cytomegalovirus (HCMV) H301 gene is cloned into the pKSSINdJRsirc plasmid, immediately downstream from the junction region core.

Briefly, HCMV strain AD169 (ATCC No. VR-538), is propagated in a permissive cell line, for example primary human foreskin fibroblasts (HFF) (GIBCO/BRL, Gaithersburg, Md.), and after evidence of cytopathologic effects, virions are purified from the cell lysate. Subsequently, HCMV DNA is purified from the virions.

The HCMV H301 gene is located between viral nucleotides 23,637 and 24,742. Isolation of the HCMV H301

gene from the viral genomic DNA is accomplished by PCR amplification, with the primer pair shown below:

HCMV H301 Forward primer (buffer sequence/Xho I site/HCMV nucleotides 23,637-23,660):

5'-TAT ATC TCC AGA TGA TGA CAA TGT GGT GTC TGA CG-3' (SEQ. ID NO.58)

HCMV H301 Reverse primer (buffer sequence/Cla I site/HCMV nucleotides 24,744-24,722):

5'-TAT ATA TCG ATT CAT GAC GAC CGG ACC TTG CG-3' (SEQ. ID NO.59)

In addition to the HCMV H301 gene complementary sequences, both primers contain a five nucleotide 'buffer sequence' at their 5' ends for efficient enzyme digestion of the PCR amplicon products. This sequence in the forward primer is followed by the Xho I recognition site, and in the reverse primer this sequence is followed by the Cla I recognition site. Thus, in the 5' to 3' direction, the HCMV H301 gene is flanked by Xho I and Cla I recognition sites. Amplification of the HCMV H301 gene from HCMV DNA is accomplished with the following PCR cycle protocol:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.17	5
72	3.5	
94	0.5	30
70	3.5	
72	10	10

Following amplification, the 1,129 bp amplicon product is purified on a 1.0% agarose gel, and subsequently digested with the Xho I and Cla I enzymes and ligated into the CIAP treated pKSSINdJRsirc plasmid, previously digested with Xho I and Cla I as described above. This clone is designated pKSSINdJRsircH301. Using the same cloning strategy, the HCMV H301 gene is inserted into all of the modified synthetic junction region vectors and all of the ELVIS vectors described in Example 3.

#### Example 5

##### EXPRESSION OF MULTIPLE HETEROLOGOUS GENES FROM SINDBIS VECTORS

The plasmid pBS-ECAT (Jang et al., *J. Virol* 63:1651, 1989) includes the 5' nontranslated region of Encephalomyocarditis virus (EMCV) from nts 260-848 of the viral genome, which contains the internal ribosome entry site (IRES). EMCV nucleotides 260-827 are amplified from pBS-ECAT by PCR, using the following primer pair.

EMCV IRES Forward primer A (For insertion next to disabled junction region in vector pKSSINBVdJIR at Apa I site):

5'-TAT ATG GGC CCC CCC CCC CCC AAC G-3' (SEQ. ID NO.60)

EMCV IRES Forward primer B (For insertion between heterologous genes terminating with Cla I sites and initiating with Nco I sites):

5'-TAT ATA TCG ATC CCC CCC CCC CCC CCA ACG-3' (SEQ. ID NO.61)

EMCV IRES Reverse Primer (To be used with either primers A or B):

5'-TAT ATC CAT GGC TTA CAA TCG TGG TTT TCA AAG G-3' (SEQ. ID NO.62)

The amplicon resulting from amplification with the forward primer A and the reverse primer is flanked by Apa I and Nco I recognition sites, inside a 5 bp 'buffer sequence'.

85

The amplicon resulting from amplification with the forward primer B and the reverse primer is flanked by Cla I and Nco I recognition sites, inside a 5 bp 'buffer sequence'. Amplification of the EMCV IRES sequence from the pBSECAT plasmid is accomplished with the following PCR cycle protocol:

Temperature (°C)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.17	
72	3.5	5
94	0.5	30
70	3.5	
72	10	1

In a similar manner, the ATG corresponding to the start codon of the heterologous gene to be inserted immediately downstream of the EMCV IRES insert is modified to contain an Nco I site (CCATGG) while the 3' end is modified to contain a Cla I site.

For insertion into the pKSSINBVdJUR vector, the 589 bp ECMV-IRES amplicon is digested with Apa I and Nco I, purified on a 1% agarose gel. The heterologous gene amplicon is digested with Nco I and Cla I and purified in a similar manner. Both fragments are ligated into the CIAP treated vector digested with Apa I and Cla I as described in example 4.

For insertion into the pKSSINBV or pKSSINBVdJRsirc vectors between heterologous genes, the 589 bp amplicon is digested with Cla I and Nco I, purified on a 1% agarose gel, and ligated into the bicistronic heterologous gene vector digested with Cla I and Nco I and treated with CIAP. In a bicistronic heterologous gene configuration, the 3' end of the upstream heterologous gene is modified to terminate in a Cla I recognition site. The ATG corresponding to the start codon of the second downstream heterologous gene to be inserted immediately downstream of the EMCV IRES insert is modified to contain an Nco I site (CCATGG). Thus, from 5' to 3', the order of components is: pKSSINBV or pKSSINBVdJRsirc-gene #1-Cla/Nco EMCV IRES gene #2-3' SIN. Insertion into all of the modified junction region vectors described in Example 2 and all of the ELVIS vectors described in Example 3 follows the strategy given here for the pKSSINBV or pKSSINBVdJRsirc vectors.

The pKSSINBVdJUR vector containing a bicistronic heterologous configuration is constructed with each of the EMCV IRES amplicons described above. The first EMCV IRES amplicon is flanked by Apa I and Nco I sites and is inserted immediately downstream of the disabled junction region at the Apa I site, as described above. This EMCV IRES sequence is followed by the first heterologous gene, which terminates in a Cla I recognition site. The first heterologous gene is followed by the second EMCV IRES sequence, using the amplicon flanked by Cla I and Nco I recognition sites. The second heterologous gene follows the second EMCV IRES sequence. Thus, from 5' to 3', the order of components is: SINBVdJUR-Apa/Nco EMCV IRES gene #1-Cla/Nco EMCV IRES gene #2-3' SIN.

The plasmid pP2-5' (Pelletier et al., *Mol. Cell Biol.* 8:1103, 1988) includes the 5' nontranslated region of the poliovirus P2/Lansing strain from nucleotides 1-1,872 of the viral genome, which contains the polio IRES. Poliovirus nucleotides 320-631 are amplified from pP2-5' by PCR, using the following primer pair:

Polio IRES Forward primer A (For insertion next to disabled junction region in vector pKSSINBVdJUR at Apa I site):

86

5'-TAT ATG GGC CCT CGA TGA GTC TGG ACG TTC CTC-3' (SEQ. ID NO.63)

Polio IRES Forward primer B (For insertion between heterologous genes terminating with Cla I sites and initiating with Nco I sites):

5'-TAT ATA TCG ATT CGA TGA GTC TGG ACG TTC CTC-3' (SEQ. ID NO.64)

Polio IRES Reverse Primer (To be used with either primers A or B):

5'-TAT ATC CAT GGA TCC AAT TTG CTT TAT GAT AAC AAT C-3' (SEQ. ID NO. 65)

The amplicon resulting from PCR with the Polio IRES forward primer A/reverse primer pair shown above is flanked by Apa I and Nco I recognition sites, inside a 5 bp 'buffer sequence'. The amplicon resulting from PCR with the Polio IRES forward primer B/reverse primer pair is shown above is flanked by Cla I and Nco I recognition sites, inside a 5 bp 'buffer sequence'. Amplification of the polio IRES sequence from the pP2-5' plasmid is accomplished with the PCR protocol shown in Example 5. In a similar manner, the ATG corresponding to the start codon of the heterologous gene to be inserted immediately downstream of the Polio IRES insert is modified to contain an Nco I site (CCATGG) while the 3' end is modified to contain a Cla I site.

For insertion into the pKSSINBVdJUR vector, the 333 bp Polio-IRES amplicon is digested with Apa I and Nco I and purified on a 1.5% agarose gel. The heterologous gene amplicon is digested with Nco I and Cla I and purified in a similar manner. Both fragments are ligated into the CIAP treated vector digested with Apa I and Cla I as described in example 4.

For insertion into the pKSSINBV or pKSSINBVdJRsirc vectors between heterologous genes, the 333 bp amplicon is digested with Cla I and Nco I, purified on a 1.5% agarose gel, and ligated into the bicistronic heterologous gene vector digested with Cla I and Nco I and treated with CIAP. In a bicistronic heterologous gene configuration, the 3' end of the upstream heterologous gene is modified to terminate in a Cla I recognition site. The ATG corresponding to the start codon of the second downstream heterologous gene to be inserted immediately downstream of the polio IRES insert is modified to contain an Nco I site (CCATGG). Thus, from 5' to 3', the order of components is: pKSSINBV or pKSSINBVdJRsirc-gene #1-Cla/Nco polio IRES gene #2-3' SIN. Insertion into all of the modified junction region vectors and all of the ELVIS vectors described in Example 3 follows the strategy given here for the pKSSINBV or pKSSINBVdJRsirc vectors.

The pKSSINBVdJUR vector containing a bicistronic heterologous configuration is constructed with each of the polio IRES amplicons described above. The first polio IRES amplicon is flanked by Apa I and Nco I sites and is inserted immediately downstream of the disabled junction region at the Apa I site, as described above. This polio IRES sequence is followed by the first heterologous gene, which terminates in a Cla I recognition site. The first heterologous gene is followed by the second polio IRES sequence, using the amplicon flanked by Cla I and Nco I recognition sites. The second heterologous gene follows the second polio IRES sequence. Thus, from 5' to 3', the order of components is: SINBVdJUR-Apa/Nco polio IRES gene #1 -Cla/Nco EMCV IRES gene #2-3' SIN.

The 220 bp BiP cDNA, corresponding to the 5' leader region of the human immunoglobulin heavy-chain binding protein mRNA, is amplified from a plasmid containing the 5' noncoding region of the BiP gene, pGEM5ZBiP5'

(provided by P. Sarnow, University of Colorado Health Sciences Center), using PCR. The sequence corresponding to BiP cDNA was determined originally in the bacteriophage lambda hu28-1 clone of the human GRP78 gene (Ting and Lee, *DNA* 7:275-286, 1988). The forward primer to be used in the PCR reaction varies, depending on the Sindbis vector into which the BiP cDNA is inserted. The reverse primer for the PCR reaction is the same for all Sindbis vectors. Amplification of the BiP cDNA sequence from pGEM5ZBiP5' from the plasmid for insertion into the Sindbis vector pKSSINBVdIJR, immediately downstream of the disabled junction region, is accomplished by amplification with the following forward primer:

5'-TAT ATG GGC CCG GTC GAC GCC GGC CAA GAC-3' (SEQ. ID NO. 66)

In addition to the BiP cDNA complementary sequences, beginning at nucleotide 12, the primer contains a five nucleotide 'buffer sequence' at its 5' end for efficient enzyme digestion of the PCR amplicon products. This sequence is followed by the Apa I recognition site.

Amplification of the BiP cDNA sequence from the pGEM5ZBiP5' plasmid for insertion into the Sindbis vectors pKSSINBV, or pKSSINBVdIJRsjrc, is accomplished by amplification with the following forward primer shown below. For these vectors, the BiP cDNA is inserted between two heterologous genes, which are placed in the region corresponding to the Sindbis structural genes.

5'-TAT ATA TCG ATG GTC GAC GCC GGC CAA GAC-3' (SEQ. ID NO. 67)

In addition to the BiP cDNA complementary sequences, beginning at nucleotide 12, the primer contains a five nucleotide 'buffer sequence' at its 5' end for efficient enzyme digestion of the PCR amplicon products. This sequence is followed by the Cla I recognition site.

The reverse primer for amplification of the BiP cDNA sequence from the pGEM5ZBiP5' plasmid for insertion into the Sindbis vectors pKSSINBVdIJR, pKSSINBV, or pKSSINBVdIJRsjrc, is:

5'-TAT ATC CAT GGT GCC AGC CAG TTG GGC AGC AG-3' (SEQ. ID NO. 68)

In addition to the BiP cDNA complementary sequences, beginning at nucleotide 12, the reverse primer contains a five nucleotide 'buffer sequence' at its 5' end for efficient enzyme digestion of the PCR amplicon products. This sequence is followed by the Nco I recognition site. Amplification of the BiP cDNA from the pGEM5ZBiP5' is accomplished with PCR protocol that are described above. In a similar manner, the ATG corresponding to the start codon of the heterologous gene to be inserted immediately downstream of the BiP IRES insert is modified to contain an NcoI site (CCATGG) while the 3' end is modified to contain a ClaI site.

For insertion into the pKSSINBVdIJR vector, the 242 bp BiP IRES amplicon is digested with Apa I and Nco I and purified on a 2% agarose gel. The heterologous gene amplicon is digested with NcoI and ClaI and purified in a similar manner. Both fragments are ligated into the CIAP treated vector digested with Apa I and ClaI as described in example 4.

For insertion into the pKSSINBV or pKSSINBVdIJRsjrc vectors between heterologous genes, the 242 bp BiP IRES amplicon is digested with Cla I and Nco I, purified on a 2% agarose gel, and ligated into the bicistronic heterologous gene vector digested with Cla I and Nco I and treated with CIAP. In a bicistronic heterologous gene configuration, the 3' end of the upstream heterologous gene is modified to terminate in a Cla I recognition site. The ATG corresponding to the start codon of the second downstream heterologous

gene to be inserted immediately downstream of the BiP cDNA insert is modified to contain an Nco I site (CCATGG). Thus, from 5' to 3', the order of components is: pKSSINBV or pKSSINBVdIJRsjrc-gene #1-Cla/Nco BiP-gene #2-3' SIN. Insertion into all of the modified junction region vectors described in Example 2, and into all of the ELVIS vectors described in example 3, follows the strategy given here for the pKSSINBV or pKSSINBVdIJRsjrc vectors.

The pKSSINBVdIJR vector containing a bicistronic heterologous configuration is constructed with each of the BiP cDNA amplicons described above. The first BiP cDNA amplicon is flanked by Apa I and Nco I sites and is inserted immediately downstream of the disabled junction region at the Apa I site, as described above. This BiP sequence is followed by the first heterologous gene, which terminates in a Cla I recognition site. The first heterologous gene is followed by the second BiP cDNA sequence, using the amplicon flanked by Cla I and Nco I recognition sites. The second heterologous gene follows the second BiP sequence. Thus, from 5' to 3', the order of components is: SINBVdIJR-Apa/Nco BiP-gene #1 -Cla/Nco BiP-gene #2-3' SIN.

Sequences which promote ribosomal readthrough are placed immediately downstream of the disabled junction region in the pKSSINBVdIJR vector, which allows ribosomal scanning in genomic mRNA from non-structural gene termination to the heterologous genes. The heterologous proteins are expressed from genomic length mRNA by ribosomal scanning. This extends the life of the infected target cell because no subgenomic transcription occurs in cells infected with this vector. Further, these same ribosomal scanning sequences are placed between heterologous genes contained in polycistronic subgenomic mRNAs. The ribosomal spanning sequence to be used in the pKSDINBVdIJR vector and between heterologous genes in the polycistronic mRNA region is:

5'-TTA ATT AAC GGC CGC CAC CAT GG-3' (SEQ. ID NO. 69)

The boldfaced codons refer to the ochre stop codon and AUG start codon, respectively. The bases underlined surrounding the stop codon refer to the Pac I recognition site and the bases underlined surrounding the start codon refer to the Nco I recognition site. The intercistronic distance of 15 bp between the start and stop codons allows efficient ribosomal readthrough, as shown previously (Levine et al., *Gene* 108:167-174, 1991). The sequences surrounding the ATG start codon from bases -9 to +1 conform to the Kozak consensus sequence for efficient translational initiation (Kozak, *Cell* 44:283-292, 1986). Where possible, the 3' terminal nucleotide corresponding to the carboxy terminal amino acid is changed to T, by site-directed mutagenesis. Also, the 5' terminal nucleotide corresponding to the amino terminal amino acid in the downstream cistron is changed to G, by site-directed mutagenesis.

Insertion of the intercistronic sequence between heterologous genes, or downstream of the disabled junction region in vector pKSDINBVdIJR, modified as described above, is accomplished by insertion of the double-stranded oligonucleotide pair shown below, into compatible Pac I/Nco I ends:

Read through sense Oligonucleotide:

5'-TAA CGG CCG CCA C-3' (SEQ. ID NO. 70)

Read through antisense Oligonucleotide:

5'-CCA TGG TGG CGG CCG TTA AT-3' (SEQ. ID NO. 71)

The oligonucleotides above are mixed in equal molar quantities in the presence of 10 mM MgCl<sub>2</sub>, heated at 95° C. for 5 min, then allowed to cool slowly to room temperature,



yielding the desired intercistronic sequence flanked by Pac I and Nco I sites. The intercistronic sequence is then ligated into the appropriate vector containing Pac I and Nco I compatible sites.

Another aspect of the present invention to enable expression of multiple heterologous genes in eukaryotic layered vector initiation systems is based on the use of alternate splicing signals. In this configuration, a splice donor sequence is inserted immediately downstream of the junction region promoter, followed by one or more heterologous genes, each of which is preceded by a splice acceptor sequence. As such, multiple splice acceptor/heterologous gene inserts may be arrayed 3' to one another. This creates a system whereby multiple heterologous genes are expressed from a single eukaryotic layered vector initiation system transcript, which is processed alternately at each splice acceptor site to give rise to individual autocatalytic RNAs encoding an individual heterologous gene. In such a system, levels of expression for each heterologous gene is controlled independently by altering the nucleotide sequence of the splice acceptor site. In addition, multiple splice donor/acceptor sites may be engineered into the system. Finally, tissue specific splice donor/acceptor sequences may be utilized in such a system to control the expression in specific tissues.

#### Example 6

##### EXPRESSION OF MULTIPLE HETEROLOGOUS GENES BY COPACKAGING

The ability to copackage multiple RNA molecules in the same alphavirus vector particle can be useful for the expression of multiple heterologous gene products from a single alphavirus vector particle. In addition, this concept can also be adapted in order to allow very large genes to be carried on RNA molecules separate from the alphavirus vector RNA containing the nonstructural genes, thus avoiding the need to package very long vector RNA molecules.

In order to accomplish such copackaging, all RNA fragments must contain a 5' sequence which is capable of initiating transcription of an alphavirus RNA, an alphavirus RNA polymerase recognition sequence for minus-strand synthesis, and at least one copy of the RNA packaging sequence. At least one of the RNA fragments also must contain sequences which code for the alphavirus non-structural proteins. Within preferred embodiments of the invention, one or more of the RNA fragments to be copackaged also will contain a viral junction region followed by a heterologous gene.

##### A. CONSTRUCTION OF COPACKAGED EXPRESSION CASSETTES FOR EXPRESSION OF MULTIPLE HETEROLOGOUS GENES

In order to demonstrate the feasibility of copackaging to allow for the expression of multiple heterologous genes, two vector constructs are created. The first construct consists of a 5' sequence that is capable of initiating transcription of Sindbis virus RNA, Sindbis RNA sequences required for packaging, sequences encoding the synthesis of nonstructural proteins 1-4, a Sindbis junction region, the luciferase gene, and Sindbis 3' sequences required for synthesis of the minus strand RNA. The second construct consists of a 5' sequence that is capable of initiating transcription of a Sindbis virus, Sindbis sequences required for packaging, a Sindbis Junction region, Sequences encoding the LacZ gene, and Sindbis 3' sequences required for synthesis of the minus strand RNA. RNA transcripts of these constructs transfected into a packaging cell line are copackaged to produce a vector

particle capable of transferring expression of both luciferase and  $\beta$ -galactosidase into the same eukaryotic cell.

The  $\beta$ -galactosidase reporter gene is inserted into the Sindbis Basic Vector (pKSSINBV) followed by deletion of a portion of the Sindbis non-structural proteins from the vector. RNA from this construct is cotransfected with RNA from Sindbis Luciferase Vector (pKSSINBV-luc) and is copackaged by one of the methods described in Example 7. Infection of fresh BHK-21 cells with vector particles containing the copackaged RNA expression cassettes should result in the expression of both luciferase and  $\beta$ -galactosidase in the same cell.

##### B. CONSTRUCTION OF A $\beta$ -GALACTOSIDASE EXPRESSION CASSETTE

KSSINBV-Linker is digested with the enzyme Sac I, which cleaves immediately after the Sindbis 3'-end and poly A sequence. The digested fragment is treated with alkaline phosphatase and purified using GeneClean. Two 12 mer oligonucleotides,

5'GGTTTAAACAGGAGCT 3' (SEQ. ID NO. 72)

5'CCTGTTTAAACCAGCT 3' (SEQ ID NO. 73)

which form the Pme I site with SacI compatible ends when hybridized, were phosphorylated and ligated into the SacI digested vector. This construct is known as pKSSINBV-Linker-PmeI. The Pme I recognition site is substituted for the Sac I site in order to create a site for linearization of the plasmid prior to SP6 transcription. The lacZ gene contains several Sac I sites. pKSSINBV-Linker-PmeI is digested with Pml I and Bcl I followed by purification with GENECLAN. The lacZ gene is obtained by digestion of pSV  $\beta$ -galactosidase vector DNA (Promega Corp., Madison, Wis.) with the enzyme HindIII. The digest is blunt-ended with Klenow DNA polymerase and dNTPs. The Klenow is heat killed and the plasmid is further digested with Bam HI and Xmn I. Xmn I reduces the size of the remaining vector fragment to simplify gel purification of the lacZ fragment. The 3.7kbp lacZ fragment is purified from a 1% agarose gel and ligated into the PmlI/Bcl I digested pKSSINBV-Linker-PmeI fragment. This construct is known as pKSSINBV-lacZ. pKSSINBV-lacZ is digested with Bsp EI and religated under dilute conditions. This results in the removal of the Sindbis nonstructural proteins between nt#422-7054. This Sindbis construct is known as pKSSINBVdINSP-lacZ.

pKSSINBVdINSP-lacZ and pKSSINBV-luc are linearized with Pme I and Sac I, respectively, and SP6 transcripts are prepared as described in Example 3. These RNA transcripts are cotransfected into packaging cells that express the Sindbis structural proteins by one of the mechanisms described in Example 7. Each RNA transcript contains a 5' sequence that is capable of initiating transcription of a Sindbis virus, RNA sequences required for packaging, a Sindbis junction region, a reporter gene, and Sindbis 3' sequences required for synthesis of the minus strand RNA. The pKSSINBV-luc transcript also contains the Sindbis non-structural proteins. In cotransfected cells, both RNA transcripts are replicated and some viral particles will contain both RNA transcripts copackaged into the same particle. Infection of fresh cells with the copackaged RNA particles will result in cell that express both luciferase and  $\beta$ -galactosidase.

##### C. COPACKAGING OF MULTIPLE EXPRESSION CASSETTES TO INCREASE PACKAGING CAPACITY

Large genes such as Factor VIII can benefit from copackaging. Briefly, insertion of the cDNA coding for Factor VIII into the Sindbis Basic Vector (pKSSINBV) results in an RNA transcript approaching 16 kb in length. Because of the increased length, this RNA cannot be replicated or packaged



efficiently. Using approaches described above, the Sindbis nonstructural proteins and the Factor VIII gene could be divided onto separate RNA molecules of approximately 8 kb and 9 kb in length, and copackaged into the same particles.

**D. CONSTRUCTION OF A FACTOR VIII EXPRESSION CASSETTE**

The pKSSINBV-Linker-PmeI construct is digested with the enzyme Bsp EI and religated under dilute conditions. This results in the removal of the Sindbis nonstructural proteins between nt#422-7054. This construct is known as pKSSINBVdINSP-Linker-PmeI. The pKSSINBVdINSP-Linker-PmeI construct is digested with the enzymes Pml I and Stu I and purified by using GeneClean. The source of Factor VIII cDNA is clone pSP64VIII, an ATCC clone under the accession number 39812 having a cDNA encoding the full-length human protein. pSP64-VIII is digested with Sal I, the ends are blunted with T4 DNA polymerase and 50 uM of each dNTP, and the ca. 7700 bp. fragment is electrophoresed on a 0.7% agarose/TBE gel and purified with GeneClean. The 7.7 kb fragment encoding Factor VIII is purified in a 0.7% agarose gel and subsequently ligated to the Pml I/Stu I digested pKSSINBVdINSP-Linker-PmeI fragment. This construct is known as pKSSINBVdINSP-Factor VIII.

pKSSINBVdINSP-Factor VIII and pKSSINBV constructs are linearized with Pme I and Sac I, respectively. SP6 transcripts are prepared as described in Example 3. These RNA transcripts are cotransfected into packaging cells that express the Sindbis structural proteins by one of the mechanisms described in Example 7. Both RNA transcripts contain a 5' sequence that is capable of initiating transcription of Sindbis RNA, sequences required for RNA packaging, a Sindbis Junction region, and the Sindbis 3' sequences required for synthesis of the minus strand RNA. In addition, the pKSSINBV transcript contains the Sindbis nonstructural protein genes, and the pKSSINBVdINSP-Factor VIII construct contains the Factor VIII gene, but not the Sindbis nonstructural protein genes. In cotransfected cells, both RNA transcripts are replicated and some viral particles will contain both RNA transcripts copackaged into the same vector particle. Infection of fresh BHK-21 cells with the copackaged RNA will result in Factor VIII expression only if both RNA molecules are present in the same cell.

#### **E. CONSTRUCTION OF AN AURA VIRUS COPACKAGING VECTOR**

To develop Aura virus expression systems analogous to those described for Sindbis, standard techniques known in the art (e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989), as well as specific approaches described herein, will be utilized for constructions. Virus, obtained from the ATCC, is propagated on cultured cells, its virion RNA extracted, and cDNA spanning the entire genome synthesized and cloned using conventional techniques. This cDNA is then used to construct gene transfer vector systems similar in principal to those described above, including, but not limited to, a replicon capable of carrying the heterologous gene(s), packaging cell lines that express the structural protein genes, and unique to this system, a separate packaging-competent subgenomic vector capable of carrying the additional heterologous gene(s). Since Aura virus subgenomic RNA contains a packaging signal, preliminary experiments are performed to identify this sequence, in order to prevent its inactivation during replacements with heterologous the gene(s). After identification of the packaging sequence, the individual elements of this Aura-based system are generated.

A basic replicon vector is constructed to contain the following minimum elements: Aura 5' sequences necessary for replication, nonstructural protein coding regions, a modified or unmodified junction region for subgenomic mRNA synthesis, a restriction enzyme site for insertion of heterologous gene(s), one or more copies of the packaging signal, and 3' Aura sequences necessary for replication, including a polyadenylate sequence. An upstream bacteriophage RNA polymerase promoter will be utilized for in vitro transcription of replicon RNA; alternatively, a eukaryotic RNA polymerase promoter will be utilized for transcription directly from cDNA.

A packaging-competent subgenomic vector is also constructed to contain the following minimum elements: a modified or unmodified junction region, a restriction enzyme site for insertion of heterologous gene(s), one or more copies of the packaging signal, and 3' Aura sequences necessary for replication/minus-strand synthesis, including a polyadenylate sequence. The subgenomic vector may, in some cases, be constructed with the Aura 5' replication sequences positioned upstream of the junction region, such that the vector will function as an amplicon. Transcription of subgenomic vector RNA can be accomplished in vitro using a bacteriophage RNA polymerase promoter, or cDNA in vivo using a eukaryotic RNA polymerase promoter. Further, the initial transcript may be of the sense-configuration or of the antisense-configuration.

Packaging cell lines are also constructed as described previously for Sindbis vectors, such that mRNA for one or more of the structural proteins will be transcribed from the junction region and be inducible by the Aura replicon. In other cases, one or more of the structural proteins can be expressed under the control of an inducible or constitutive eukaryotic promoter. In each case, specific inactivating mutations are made in any packaging sequences present in the structural protein genes, in order to prevent encapsidation of these sequences with the replicon. These mutations should be silent changes, usually at the third position of the codon, which do not affect the amino acid encoded.

The ability to package multiple heterologous genes can be exploited for many therapeutic applications, which include, but are not limited to, expression of multiple cytokines, multiple CTL epitopes, combinations of cytokines and CTL epitopes to enhance immune presentation, multiple subunits of a therapeutic protein, combinations of therapeutic proteins and antisense RNAs, etc. In addition to its utility for the expression of multiple heterologous genes, the packaging of subgenomic mRNAs into virions also enables this vector system for the transfer of extremely long heterologous sequences. Furthermore, this multipartite approach is useful in the development of producer cell lines, wherein replicase proteins and structural proteins are being stably expressed, and any heterologous gene contained within a subgenomic vector could then be readily introduced as a stable integrant.

#### **Example 7**

##### **CONSTRUCTION OF ALPHAVIRUS PACKAGING CELL LINES**

##### **A. SELECTION OF PARENT CELL LINES FOR ALPHAVIRUS PACKAGING CELL LINE DEVELOPMENT**

##### **1. PERSISTENTLY OR CHRONICALLY INFECTABLE CELLS**

An important criteria in selecting potential parent cell lines for the creation of alphavirus packaging cell lines, is the choice of cell lines that exhibit little or no cytopathological effects, prior to the appropriate production of

alphavirus vector particles. This criteria is essential for the development of an alphavirus vector producer cell line which can be propagated for long periods of time and used as a stable source of vector. It is known that alphavirus infection of most mammalian cells results in cytopathology and lysis of the cell. However, the derivation of packaging cells from various insect cell lines may circumvent this problem. For example, insect cell lines, such as *Aedes albopictus*, *Aedes aegypti*, *Spodoptera frugiperda*, and *Drosophila melanogaster* cells, may be utilized to construct packaging cell lines. For example, within one embodiment, alphavirus packaging cell lines are provided using an configuration uses an insect parent cell line, such as the *Aedes albopictus*, containing a stably transfected expression cassette vector which allows for expression of alphavirus structural proteins under the control of inducible or non-inducible promoters active in these cell types, and co-expressing a selectable marker.

Recently, a Sindbis virus-induced protein of cellular origin, which has been associated with the down-regulation of Sindbis virus production in some infected *Aedes albopictus* cells, has been identified and purified and Brown, *Virology* 194(1):44-49, 1993. The protein is a small hydrophobic peptide of approximately 3200 Da., which can induce an antiviral state and inhibit both 49S and 26S viral RNA synthesis. Cells treated with the antiviral peptide usually demonstrate quiescent arrest of cellular division for 96 hours in uninfected cells, and then normal growth rates are restored. Cells that have been exposed to this peptide prior to infection are unable to replicate Sindbis virus and appear to maintain this phenotype by constitutively producing the antiviral protein through 10 months of continuous passage.

It is recognized that this cellular response to Sindbis replication in *Aedes albopictus* cells might decrease the efficiency of a recombinant alphavirus vector producing system in those cells. To improve the efficiency of alphavirus vector production, two methods have been devised to inactivate the virus-induced cellular antiviral protein, thus preventing any reduction of vector particle titers. The first method entails purification of this cellular protein described above, and determination of a portion of the primary amino acid sequence using established techniques known in the art. The resulting amino acid sequence is then used to derive possible corresponding genomic sequences, enabling one to design a degenerate PCR primer pair which can be used to amplify the specific cellular sequence. This amplified sequence is then cloned using standard techniques known in the art, to obtain a discreet region of the gene encoding this inhibitory protein. Determination of the nucleotide sequence of this clone then enables one to design a vector which will integrate specifically within this Sindbis inhibitory gene by homologous recombination, and "knock out" its capacity to express a functional protein. Cell clones which contain the knock out sequence are identified by insertion of a selectable marker into the discreet cloned region of the inhibitory protein, prior to transfecting cells with the vector.

A second method for disabling this Sindbis virus inhibitory protein involves the treatment of *Aedes albopictus*-derived packaging cells with a mutagen, for example, BUdR (5-bromodeoxyuridine). The mutagenized packaging cell line population is then transfected or transduced with a Sindbis vector, which is able to express the neomycin resistance marker. Under high concentrations of the G418 drug, only those cells producing large amounts of Sindbis vector, and thus unable to express the Sindbis inhibitory gene, will be able to survive. After selection, resistant

colonies are pooled, dilution cloned, and tested for high titer Sindbis production.

## 2. MODIFICATION OF CELLS TO DECREASE SUSCEPTIBILITY TO ALPHAVIRUS EXPRESSION: SUPPRESSION OF APOPTOSIS AND CYTOPATHOLOGY

Packaging cell lines may also be modified by overexpressing the bcl-2 gene product in potential parent cell lines, such as canine D-17 and Cf2; human HT1080 and 293; quail QT-6; baby hamster kidney BHK-21; mouse neuroblastoma N18; and rat prostatic adenocarcinoma AT-3. The conversion of these cells to a persistently injectable state allows for their use as alphavirus packaging and producer cell lines, similar to those of retrovector producer lines.

In order to construct such packaging cells, a bcl-2 expression vector is constructed by using standard recombinant DNA techniques in order to insert the 910 base pair Eco RI cDNA fragment derived from the plasmid p84 (*Nature* 336:259) into any commercially available expression vector containing a constitutive promoter and encoding a selectable marker, for example, pCDNA3 (Invitrogen, San Diego, Calif.). Careful consideration must be taken to avoid any type of homology between alphavirus nucleic acid sequences and other transduced vectors. This precaution should be taken in order to prevent recombination events which may lead to undesirable packaging of selectable markers or the bcl-2 oncogene in recombinant Sindbis particles. This is an important point, since the alphavirus vector system described herein is designed for use as a biological therapeutic. Once the bcl-2 expression vector is constructed, the parent cell line (i.e., BHK-21 cells) is transfected using any standard technique and selected after 24 hours using the appropriate marker. Resistant colonies are pooled, followed by dilution cloning, and then individual clones are propagated and screened for bcl-2 expression. Once expression is verified, persistent Sindbis infection is tested, followed by its use as a parent cell line for alphavirus packaging cell line development.

Other gene products, in addition to the bcl-2 oncogene, which suppress apoptosis may likewise be expressed in an alphavirus packaging or producer cell line. Three viral genes which are particularly preferred include: the adenovirus E1B gene encoding the 19-kD protein (Rao et al., *PNAS* 89:7742-7746, 1992), the herpes simplex virus type 1, 34.5 gene (Chou and Roizman, *PNAS* 89:3266-3270, 1992), and the AcMNPV baculovirus p35 gene (Clem et al., *Science* 254:1388-1390, 1991). These individual genes may be inserted into any commercially available plasmid expression vectors, under the control of appropriate constitutive eukaryotic transcriptional promoters, and also containing a selectable marker, using standard techniques. The expression vector constructs are subsequently transfected into cell lines as described above, and the appropriate selection is applied. Selection for stable integration of these genes and constitutive expression their products should allow for more extended vector production in cell lines found to be susceptible to alphavirus-induced apoptotic events. In addition, it is feasible that each gene product inhibits apoptosis by its own unique mechanism. Therefore, the genes may also be introduced into packaging or producer cell lines in various combinations in order to obtain a stronger suppressive effect. Finally, other gene products having similar effects on apoptosis can also be readily incorporated into packaging cell lines as they are identified.

In the derivation of alphavirus vector packaging and producer cell lines, many approaches are outlined to control the expression of viral genes, such that producer cell lines stably transformed with both vector and vector packaging

cassettes, can be derived. These approaches include inducible and/or cellular differentiation sensitive promoters, antisense structural genes, heterologous control systems, and mosquito or other cells in which viral persistent infections are established. Regardless of the final configuration for the alphavirus vector producer cell line, the ability to establish persistent infection, or at least delay cell death as a result of viral gene expression, may be enhanced by inhibiting apoptosis. For example, the DNA tumor viruses, including adenovirus, HPV, SV40, and mouse polyomavirus (Py), transform cells in part, by binding to, and inactivating, the retinoblastoma (Rb) gene product p105 and its closely related gene product, p107, and other gene products involved in the control of the cell cycle including cyclin A, p33<sup>cdk2</sup> and p34<sup>cdk2</sup>. All of these viruses, except for Py, encode gene products which bind to and inactivate p53. Uniquely, Py encodes middle T antigen (mT) which binds to and activates the membrane tyrosine kinase, src, and also phosphatidylinositol-3-kinase, which is required for the full transformation potential of this virus (Talmage et al., *Cell* 59:55-65, 1989). The binding to and inactivation of the Rb and p53 recessive oncogene products prevents cells transformed by these DNA tumor viruses from entering the apoptotic pathway. It is known that p53 is able to halt the division of cells, in part by inhibiting the expression of proteins associated with cellular proliferation, including c-fos, hsc70, and bcl-2 (Miyashita et al., *Cancer Research* 54:3131-3135, 1994).

In order to extend the duration of alphavirus vector production, or to promote a persistently infectable state, packaging and producer cells are transformed with viral genomic DNA from Py or SV40. In particular, SV40 and Py transformed cell lines are established, and the kinetics and level of Sindbis production and cytopathology after viral infection determined. If apoptotic events characteristic of Sindbis proliferation in hamster cells are diminished, each prototype alphavirus packaging and producer cell line subsequently is transformed with Py or SV40, in order to increase the yield of packaged vector from these cells.

### 3. MODIFICATION OF CELLS TO DECREASE SUSCEPTIBILITY TO ALPHAVIRUS EXPRESSION: PRODUCTION OF ACTIVATION-DEPENDENT VECTOR PARTICLES

The Sindbis E2 glycoprotein is synthesized as a precursor, PE2. This PE2 precursor along with the second viral glycoprotein, E1, associate in the endoplasmic reticulum and are processed and transported to the infected cell membrane as a heterodimer for virion incorporation. At some point during this processing, PE2 is cleaved into E3 and the mature virion glycoprotein E2. E3 is the 64 amino-terminal residues of PE2 and is lost in the extracellular void during maturation. The larger cleavage product, E2, is associated with E1 and anchored in what becomes the viral envelope. Host cell protease(s) is responsible for processing of the PE2 precursor, cleaving at a site that immediately follows a highly conserved canonical four amino acid (aa) residue motif, basic-X-basic-basic aa's. A mutant cell line derived from the CHO-K1 strain, designated RPE.40 (Watson et al., *J. Virol* 65:2332-2339, 1991), is defective in the production of Sindbis virus strain AR339, through its inability to process the PE2 precursor into the E3 and mature E2 forms. The envelopes of Sindbis virions produced in the RPE.40 cell line therefore contain a PE2/E1 heterodimer. RPE.40 cells are at least 100-fold more resistant to Sindbis virus infection than the parental CHO-K1 cells, suggesting an inefficiency in the ability of PE2 containing virions to infect these cells. The defective virions produced by the RPE.40

cell line can be converted into a fully infectious form by treatment with trypsin.

In packaging and producer cell lines, any wild-type alphavirus that is produced by recombination between vector and structural protein gene RNAs will re-infect cells and be rapidly amplified; thus, significantly contaminating and decreasing the titer of packaged vector preparations. Packaging and producer cells developed from the RPE.40 line are an alternative to other cell lines permissive for alphavirus infection due to the inefficient amplification of any wild-type virus generated during vector production and packaging. Thus, vector preparations are not significantly contaminated with wild-type virus. Furthermore, the benefits of this system are extended to other packaging and producer cell lines by developing "knock-out" mutants in their analogous cellular protease(s), using techniques known in the art.

### 4. HOPPING CELL LINE DEVELOPMENT

Alphavirus hopping cell lines, as discussed previously, are used transiently to produce infectious RNA vector particles which have been pseudotyped for a different cellular receptor tropism. Once the hopping cell line produces vector particles, it is no longer required because only the infectious culture supernatants are needed to transduce the original alphavirus packaging cell lines discussed above. Therefore, the hopping cell line need not exhibit persistent infection by alphavirus in order to transiently produce vector particles. In this instance, the parent cell line can be either an insect cell line that exhibits persistent infection, or a mammalian cell line which is likely to lyse within 24-72 hours after a productive alphavirus infection. The only criteria is that the cell lines are able to express either VSV-G protein, with or without the appropriate alphavirus structural proteins, or retroviral gag-pol and env protein without affecting cell growth prior to introduction of the alphavirus RNA vector. Therefore, the alphavirus hopping cell line can be any of the aforementioned parent cell lines able to support either alphavirus or retroviral replication, without the additional cell modifications discussed previously, such as bcl-2 oncogene expression.

The generation of VSV-G pseudotyped alphavirus vector particles can be accomplished by at least three alternative approaches, two of which are dependent on the stable integration of a VSV-G expression cassette into cells. VSV-G protein is known to be highly cytotoxic when expressed in cells. Therefore, synthesis of this protein by the expression cassette is controlled by an inducible promoter. Specifically, a DNA fragment containing the VSV-G protein gene is isolated from plasmid pLGRNL (Emi et al., *J. Virol.* 65:1202-1207, 1991) by digestion with Bam HI, the termini made blunt using Klenow fragment enzyme and dNTPs, and the 1.7 kb fragment purified from a 1% agarose gel. Plasmid vector pVGELVIS-SINBV-linker (from Example 3), is digested with the enzyme Bsp EI to remove Sindbis non-structural protein coding sequences nts. 422-7054, and the remaining vector is re-ligated to itself to generate plasmid pVGELVISdINSP-BV-linker. This plasmid is then digested with Xho I and the termini made blunt using Klenow fragment enzyme and dNTPs. The previously purified VSV-G fragment is subsequently ligated with this vector DNA, and resulting clones are screened for proper VSV-G insert orientation. This pVGELVIS-based VSV-G expression construct, in which VSV-G synthesis is controlled by a Sindbis replicon-inducible junction region, is designated pVGELVISdI-G.

Alternatively, a similar Sindbis replicon-inducible VSV-G expression cassette may be generated in the antisense configuration. In particular, plasmid vector pKSSINBV-linker

(described in Example 3) is digested with the enzymes Apa I and Bam HI to most of the Sindbis nonstructural protein coding region, and the resulting 3309 bp vector fragment is purified from a 1% agarose gel. In addition, plasmid pd5'-26s (described in section B.3., this example) also is digested with the enzymes Apa I and Bam HI. The resulting 400 bp fragment which contains the HDV ribozyme/Sindbis 5'-end fusion is purified from a 1% agarose gel and subsequently ligated with the purified pKSSINBV-linker vector fragment to generate a plasmid designated pd5'-BVlinker. Plasmid pd5'-BVlinker is subsequently digested with Xho I, the termini made blunt using Klenow fragment enzyme and dNTPs, and ligated with the previously purified VSV-G fragment. The resulting construct, containing the expression cassette elements HDV antigenomic ribozyme/Sindbis 5'-end 299 nts./Sindbis junction region/VSV-G protein gene/Sindbis 3'-end untranslated region, is designated as plasmid pd5'-BV-G. Insertion of this VSV-G gene cassette into the pcDNA3 vector is as follows. Plasmid pd5'-BV-G is digested with the enzymes Pme I and Apa I, and the termini are made blunt by the addition of T4 DNA polymerase and dNTPs. The entire 2.5 kb VSV-G protein gene cassette is purified in a 1% agarose gel. Plasmid pcDNA3 is digested with the enzymes HindIII and Apa I and the termini are made blunt by the addition of T4 DNA polymerase and dNTPs, and the 5342 bp vector is purified in a 1% agarose gel. The two purified, blunt-end DNA fragments are subsequently ligated, and the resulting VSV-G protein gene expression cassette vector is known as plasmid pCMV/d5'VSV-G. Further modifications of the VSV-G expression cassettes pVGELVISdl-G and pCMV/d5'VSV-G to substitute other selectable markers, for example hygromycin resistance or E. coli gpt, for the current neomycin resistance, or other promoter elements, for example Drosophila metallothionein or hsp 70, for the current CMV, MuLV, and SV40 promoters, may be readily accomplished given the disclosure provided herein.

In a first VSV-G/alphavirus hopping cell line configuration, VSV-G expression cassette plasmid DNA (pVGELVISdl-G or pCMV/d5'VSV-G, or modified versions thereof) is transfected into the appropriate cell type (for example, BHK-21 cells) and selection for G418 resistance is applied using media containing 400 g/ml of G418 as described elsewhere in this example. G418-resistant cells are cloned by limiting dilution and the individual cell lines expanded for screening. VSV-G expressing cell lines are detected by transfection with any nonstructural protein gene-containing RNA vector (see Example 3) to induce the VSV-G expression cassette, followed by immunofluorescence using polyclonal rabbit anti-VSV antibody as described (Rose and Bergmann, *Cell* 34:513-524, 1983). The stably transfected VSV-G expressing cell line, in some cases, is subsequently transfected with plasmid expression cassette(s) which express one or more Sindbis structural proteins (described elsewhere in this example). For the production of VSV-G pseudotyped alphavirus particles, the appropriate vector RNA is transfected into the VSV-G hopping cell line, and vector particle-containing supernatants are recovered at least 24 hours post-transfection.

In a second VSV-G/alphavirus hopping cell line configuration, VSV-G expression cassette DNA (pVGELVISdl-G or pCMV/d5'VSV-G, or modified versions thereof) is transfected into previously derived alphavirus packaging cell lines (described elsewhere in this example) and the appropriate selection is applied as described previously. The selected cells are cloned by limiting dilution and the individual cell lines expanded for screening. VSV-G

expressing cell lines are detected by transfection with any nonstructural protein gene-containing RNA vector (see Example 3) to induce the VSV-G expression cassette, followed by immunofluorescence using polyclonal rabbit anti-VSV antibody as described (Rose and Bergmann, *Cell* 34:513-524, 1983). For the production of VSV-G pseudotyped alphavirus particles, the appropriate vector RNA is transfected into the VSV-G hopping cell line, and vector particle-containing supernatants are recovered at least 24 hours post-transfection.

In a third VSV-G/alphavirus hopping cell line configuration, VSV-G expression cassette DNA is co-transfected with the appropriate vector RNA into previously derived alphavirus packaging cell lines (described elsewhere in this example). Supernatants containing pseudotyped vector particles are recovered at least 24 hours post-transfection.

For the pseudotyping of alphavirus vectors in retroviral packaging cell lines, any cell line referenced in the literature, which expresses retroviral gag-pol and env sequences, may be used to package alphavirus RNA vector that has been engineered to contain a retroviral packaging sequence. The retrovirus psi packaging sequence is inserted between the inactivated junction region and a synthetic junction region tandem repeat, such that only genomic-length vector, and not subgenomic RNA, is packaged by the retroviral envelope proteins. Retroviral-based particles containing alphavirus vector RNA are produced by transfecting in vitro transcribed alphavirus vector RNA using procedures that have been described previously. Supernatants with pseudotyped retroviral particles containing alphavirus RNA vector are harvested at 24 hours post-transfection, and these supernatants are then used to transduce an alphavirus packaging cell line.

##### 5. IDENTIFICATION OF PARENT CELL LINES WHICH PRODUCE ALPHAVIRUS RESISTANT TO INACTIVATION BY HUMAN COMPLEMENT

Successful intravenous administration of recombinant alphavirus particles requires that the vector is resistant to inactivation in serum. It is well known to those skilled in the art that Sindbis grown on BHK cells is sensitive to inactivation, in terms of effective virus titer. In order to identify parent cell lines which produce Sindbis particles which are resistant to inactivation by human complement, the level of serum inactivation of Sindbis virus grown on multiple cell types is tested. The cell types tested are derived from many species, including human, for example, 293 or HT1080 (ATCC No. CCL 121).

As a source of human complement, approximately 70 mls of blood are collected from patients into serum separating tubes (Becton Dickinson, Los Angeles, Calif.). The blood is allowed to clot for one half hour at room temperature. After clotting the serum is centrifuged at 2000 g for 10 minutes at 4° C. The serum is collected and placed into a 15 ml conical tube (Corning, Corning, N.Y.) and placed on ice. Approximately, 1.1 ml aliquots of the serum are placed in 2 ml cryovials, frozen in a dry ice/ethanol bath and stored at -70° C. for subsequent serum inactivation assays. Complement inactivated controls are prepared by heat inactivation of control aliquots for 30 minutes at 56° C.

To test Sindbis for serum inactivation, two vials containing 1.1 ml of 100% non-heat inactivated human serum are used for various virus preparations. One vial of serum is quick thawed at 37° C. The serum is then heated to 56° C. for 30 minutes to heat inactivate complement present in the serum. Following inactivation the serum is placed on ice. The second vial is quick thawed at 37° C. After thawing the serum is placed on ice.

Approximately, 1.0 ml of the non-heat inactivated serum, medium, and heat-inactivated serum are placed in separate 1.5 ml tubes (Fisher Scientific, Pittsburgh, Pa.) and mixed with 10<sup>5</sup> Plaque Forming units (PFU) of Sindbis virus and incubated at 37° C. for 1 hour. After incubation the tubes are placed on ice.

In order to identify the parent cell line host from which an alphavirus is resistant to human serum inactivation, the non-heat inactivated serum, medium, and heat-inactivated serum virus preparations are titered by plaque assay on BHK cells. Equivalent virus titers regardless of incubation with non-heat inactivated serum, medium, or heat-inactivated serum, are indicative of parent cell line hosts from which Sindbis virus is resistant to human complement inactivation.

## B. STRUCTURAL PROTEIN EXPRESSION CONSTRUCTS

### 1. INDUCIBLE AND CONSTITUTIVE STRUCTURAL PROTEIN VECTOR EXPRESSION CASSETTES

The development of alphavirus packaging cell lines is dependent on the ability to synthesize high intracellular levels of the necessary structural proteins: capsid, pE2 and/or E2, and E1. Unfortunately, high level expression of these proteins, in particular, the envelope glycoproteins E2 and E1, may lead to concomitant cytopathology and eventual cell death. Therefore structural protein expression cassettes have been designed with inducible regulatory elements which control the levels of gene expression, in addition to others which maintain constitutive levels of expression.

In a first configuration, expression of the alphavirus structural proteins is under control of the RSV LTR, in conjunction with the inducible lac operon sequences. This is achieved by insertion of alphavirus cDNA corresponding to the viral structural protein genes into the pOP13 and pOPRSV1 vectors (Stratagene). These vectors, used separately, are co-transfected with the p3'SS vector (Stratagene), which expresses the lac repressor "i" protein. In the absence of inducer, for example, Isopropyl-B-D-thiogalactopyranoside (IPTG), the basal, or constitutive, level of expression of a luciferase reporter gene has been reported to be 10–20 copies per cell. Addition of IPTG, results in a conformational change of the repressor protein, which results in decreased affinity of the lac i protein for lac-operator sequences, permitting high level expression of the heterologous gene. Induction levels in the presence of IPTG of 95-fold have been reported for heterologous genes contained in the pOP13 vector.

Specifically, the Sindbis structural protein gene (SP) cDNA is inserted into the pOP13 and pOPRSV1 vectors as follows. The SP coding region is amplified in toto with a primer pair whose 5' ends map, respectively, to the authentic AUG translational start and UGA translational stop sites, including the surrounding nucleotides corresponding to the Kozak consensus sequence for efficient translational initiation at Sindbis nt 7638. The forward primer is complementary to Sindbis nts 7638–7661, and the reverse primer is complementary to Sindbis nts 11,384–11,364. PCR amplification of Sindbis cDNA corresponding to the structural protein genes is accomplished by a standard three-temperature cycling protocol, using the following oligonucleotide pair:

Forward primer (7638F):

5'-TATATGCGGCCGACACCACCATGAATAGAGGATTCTTTAACATGC-3' (SEQ. ID NO. 74)

Reverse primer (11384R):

5'-TATATGCGGCCGCTCATCTTCGTGTGCTAGTCAG-3' (SEQ. ID NO.75)

In addition to their respective complementaries to the indicated Sindbis nts, a 5 nucleotide "buffer sequence" followed by the Not I recognition sequence is attached to the 5' ends of each primer. Following PCR amplification, the 3,763 bp fragment is purified in a 1% agarose gel, then subsequently digested with the Not I enzyme. The resulting 3,749 bp fragment is then ligated, separately, into the pOP13 and pOPRSV1 vectors, which are digested with Not I and treated with calf intestine alkaline phosphatase. These expression cassette vectors, which contain the entire coding capacity of the Sindbis structural proteins are known as pOP13-SINSP and pOPRSV1-SINSP.

Variations of the lac operon-Sindbis structural protein gene expression cassettes also can be constructed using other viral, cellular or insect-based promoters. Using common molecular biology techniques known in the art, the lac operon and the RSV LTR promoter, or just the RSV LTR promoter, sequences can be switched out of the Stratagene pOP13 and pOPRSV1 vectors and replaced by other promoter sequences, such as the cytomegalovirus major immediate promoter (pOPCMV-SINSP); the adenovirus major late promoter (pOPAMP-SINSP); the SV40 promoter (pOPSV-SINSP); or insect promoter sequences, which include the Drosophila metallothionein inducible promoter (pMET-SINSP), Drosophila actin 5C distal promoter (pOAS5C-SINSP), heat shock promoters HSP65 or HSP70 (pHSP-SINSP), or the baculovirus polyhedrin promoter (pPHED-SINSP).

### 2. MODIFICATION OF CASSETTES TO INCREASE PROTEIN EXPRESSION LEVELS

Alphavirus structural protein expression can be increased if the level of mRNA transcripts is increased. Increasing the level of mRNA transcripts can be accomplished by modifying the expression cassette such that alphavirus nonstructural proteins recognize these transcripts, and in turn, replicate the message to higher levels. This modification is performed by adding the wild-type minimal junction region core (nucleotides 7579 to 7602) to the extreme 5'-end of the Sindbis structural protein coding region, prior to the first authentic ATG start site for translation and inverting the expression cassette in the vector, so as to produce antisense structural protein gene transcripts. This can be accomplished by following the same PCR amplification technique described above for placing the Sindbis structural protein cDNA into the pOP13 and pOPRSV1 expression vectors. The only modification to this procedure is the replacement of the 7638F forward primer with a similar primer that includes junction region core nucleotides 7579–7602 between the Not I restriction enzyme site and the first ATG of the coding region as follows:

Forward primer (JUN7638F):

5'-TATATGCGGCCGCGCATCTCTACGGTGGTCTTAATAGTACCACCACCATGAATAGAGGATTTC-3' (SEQ. ID NO. 76)

Following PCR amplification, the resulting 3,787 bp fragment is purified in a 1% agarose gel, then subsequently digested with the Not I enzyme. The resulting 3,773 bp fragment is then ligated, separately, into the pOP13 and pOPRSV1 vectors which are digested with Not I and treated with calf intestine alkaline phosphatase. The resulting expression cassette vectors are known as pOP13-JUNSINSP and pOPRSV1-JUNSINSP. However, it must be stated that the introduction of junction region sequences into the structural protein expression cassettes will introduce sequences which may possibly lead to undesirable recombination events, leading to the generation of wild-type virus.

### 3. INDUCIBLE EXPRESSION OF STRUCTURAL PROTEINS VIA ALPHAVIRUS VECTOR

Because of potential cytotoxic effects from structural protein expression, the establishment of inducible packaging cell lines which express even modest basal levels of these proteins may not always be preferred. Therefore, packaging cell line expression cassettes are constructed which contain regulatory elements for the high level induction of structural protein synthesis via nonstructural proteins supplied in trans by the alphavirus vector, but with no basal level of synthesis until appropriately stimulated.

In this configuration, a structural protein gene cassette is constructed, whereby transcription of the structural protein genes occurs from an adjacent alphavirus junction region sequence. The primary features of this cassette are: an RNA polymerase II promoter positioned immediately adjacent to alphavirus nucleotide 1, such that transcription initiation begins with authentic alphavirus nucleotide 1, the 5'-end alphavirus sequences required for transcriptase recognition, the alphavirus junction region sequence for expression of the structural protein gene mRNA, the alphavirus structural protein gene sequences, the 3'-end alphavirus sequences required for replication, and a transcription termination/polyadenylation sequence. Because of an upstream open-reading frame which ends in translation termination codons prior to the AUG start site of the structural protein genes, expression of the alphavirus structural proteins can occur only after the synthesis of minus-strand RNA by vector-supplied nonstructural proteins, followed by the subsequent transcription of a structural protein gene mRNA from the junction region. Therefore, the inducibility of this system is dependent entirely on the presence of nonstructural proteins, supplied by the alphavirus vector itself, introduced as either RNA transcribed *in vitro*, or cDNA positioned downstream of an appropriate promoter element. In addition, the 5'- and 3'-end alphavirus sequences allow for this RNA transcript of the structural protein gene cassette to be amplified by the same vector-supplied nonstructural proteins (see FIG. 11).

Specifically, the construction of a positive-sense, vector-inducible Sindbis packaging cassette is accomplished as follows. Briefly, the pVGELVIS vector described previously is digested with the enzyme Bsp EI to remove nucleotides 422 to 7054, including most of the nonstructural gene coding sequences, and the remaining 9925 bp fragment is purified in a 0.8% agarose gel, and subsequently re-ligated to itself to generate the construct known as pLTR/SindlBspE (FIG. 11). This deletion leaves the 5'-end authentic translation start codon at nts 60-62 intact, and creates in-frame downstream UAA and UGA stop codons at nts 7130-7132 and 7190-7192 (original numbering), respectively, thus preventing translation of the downstream structural protein gene open-reading frame. The pLTR/SindlBspE packaging cassette construct is subsequently transfected into BHK cells (ATCC #CCL 10) and transfectants are selected using the G418 drug at 400 ug/ml and cloned by limiting dilution. After expansion of the transfected clonal lines, screening for packaging activity is performed by transfection of Sindbis-luciferase (Sin-luc) vector RNA as described previously. The data shown in FIG. 12 demonstrate that transfection of Sin-luc vector RNA into several of these clonal LTR/SindlBspE packaging cells results in the production of infectious Sindbis particles containing the Sin-luc RNA, as the recovered supernatants are shown to transfer Sin-luc vector RNA to fresh monolayers of BHK cells.

A similar packaging construct can also be made using the pVG-ELVISd clone (described previously) as initial material for creation of the Bsp EI deletion. In this clone, the

Sindbis 3'-end sequence is followed by a catalytic ribozyme sequence to allow more precise processing of the primary transcript adjacent to the 3'-end sequences of Sindbis. In addition, a wide variety of variations of these packaging cassette constructions can be made given the disclosure provided herein, including for example, the substitution of other RNA polymerase promoters for the current MuLV LTR, the addition of 1 or more nucleotides between the RNA polymerase promoter and the first Sindbis nucleotide, the substitution of other ribozyme processing sequences, or the substitution of a non-Sindbis-encoded open reading frame upstream of the structural protein gene sequences, which may or may not retain the 5'-end Sindbis sequences required for transcriptase recognition. Furthermore, these constructs can be transfected into other cell lines, as discussed previously.

In another vector-inducible packaging configuration, expression cassettes contain a cDNA copy of the alphavirus structural protein gene sequences flanked by their natural junction and 3'-untranslated regions, and are inserted into an expression vector in an orientation, such that primary transcription from the promoter produces antisense structural protein gene RNA molecules. Additionally, these constructs contain, adjacent to the junction region, alphavirus 5'-end sequences necessary for recognition by the viral transcriptase, and a catalytic ribozyme sequence positioned immediately adjacent to alphavirus nucleotide 1 of the 5'-end sequence. As such, this ribozyme cleaves the primary RNA transcript precisely after the first alphavirus nucleotide. In this antisense orientation, the structural protein genes cannot be translated, and are dependent entirely on the presence of alphavirus virus nonstructural proteins for transcription into positive-strand mRNA, prior to their expression. These nonstructural proteins again are provided by the alphavirus vector itself. In addition, because this configuration contains the precise alphavirus genome 5'- and 3'-end sequences, the structural protein gene transcripts undergo amplification by utilizing the same nonstructural proteins provided by the alphavirus vector.

Specifically, the Sindbis structural protein gene cDNA is removed from the genomic clone pVGSP6GEN and inserted into the pcDNA3 (Invitrogen Corp., San Diego, Calif.) expression vector as follows. First, plasmid pVGSP6GEN is digested with the enzymes Apa I and Bam HI to remove all Sindbis sequences through nucleotide 7335, including the genes encoding nonstructural proteins 1, 2, 3, and most of 4. The remaining 7285 bp vector fragment, which contains the Sindbis structural protein genes, is purified in a 0.8% agarose gel, and subsequently ligated with a polylinker sequence, called SinMCS, that is obtained by annealing two synthetic oligonucleotides. The oligonucleotides, SinMCSI and SinMCSII, contain the recognition sites for Cla I, Bgl II, and Spe I, and have Apa I and Bam HI ends after annealing. Their sequences are as follows:

SinMCSI

5'-CTCATCGATCAGATCTGACTAGTTG-3' (SEQ. ID NO. 77)

SinMCSII

5'-GATCCAAGTATGATCTGATCGATGAGGGCC-3' (SEQ. ID NO. 78)

The resulting construct, known as pMCS-26s, is then modified to contain the 5'-end 299 nucleotides of Sindbis, fused to an 84 nucleotide ribozyme sequence from the antigenomic strand of hepatitis delta virus (HDV) (*Nature* 350:434), using overlapping PCR amplification. Two primer pairs are used initially in separate reactions, followed by their overlapping synthesis in a second round of PCR. In

reaction #1, the forward primer (HDV49-XC) is complementary to HDV genome nucleotides 823-859, and the reverse primer (HDV17-68) is complementary to HDV genome nucleotides 839-887, with sequences as follows: Forward primer (HDV49-XC)

5'-ACTTATCGATGGTTCTAGACTCCCTTAGCCAT-  
CCGAGTGGACGTGCGTCCTCCTC-3' (SEQ. ID NO. 79)

Reverse primer (HDV17-68)

5'-TCCACCTCCTCGCGTCCGACCTGGGCATCC-  
GAAGGAGGACGCACGTCCACT-3' (SEQ. ID NO. 80)

In addition to their respective complementarities, primer HDV49-XC contains flanking Xba I and Cla I recognition sequences at the 5'-end. PCR amplification of HDV sequences is accomplished by a standard three-temperature cycling protocol with these primers and Vent polymerase. In reaction #2, the forward primer (SIN-HDV), which joins precisely the HDV and Sindbis sequences, is complementary to nucleotides 1-21 of Sindbis, and genomic nucleotides 871-903 of HDV, and overlaps the sequence of primer HDV17-68 (from above) by 20 nucleotides, and the reverse primer (SIN276-SPE) is complementary to Sindbis nucleotides 299-276, with sequences as follows:

Forward primer (SIN-HDV)

5'-TCGGACCGGAGGAGGTGGAGATGCCATGC-  
CGACCCATTGACGGCTAGTACACACT-3' (SEQ. ID NO. 81)

Reverse primer (SIN276-SPE)

5'-CTGGACTAGTAACTAGTGGTCTCGGAAAAC-  
ATTCT-3' (SEQ. ID NO. 82)

In addition to their respective complementarities, primer SIN276-SPE contains a flanking UAA translation termination codon and SpeI recognition sequence at its 5' end. PCR amplification of the fragment containing Sindbis 5'-end sequences fused to HDV ribozyme sequences is accomplished by a standard three-temperature cycling protocol, using Vent polymerase, these primers, and pVGSP6GEN plasmid as template. After the first round of PCR amplification, 1/2 of the total amounts from each of reaction #1 and reaction #2 is combined and used as template in a second round of PCR amplification with additional input of primers HDV49-XC and SIN276-SPE and a standard three-temperature cycling protocol. Following the second round of PCR, the 414 bp amplicon is purified with the MERMAID KIT (Bio101, La Jolla, Calif.), and digested with the enzymes ClaI and SpeI. The digested amplicon is purified in a 1% agarose gel, and subsequently ligated into plasmid pMCS-26s, which also is digested with ClaI and SpeI and purified in a 1% agarose gel. The resulting construct, containing the expression cassette elements HDV antigenomic ribozyme/Sindbis 5'-end 299 nts/Sindbis junction region/Sindbis structural protein genes/Sindbis 3'-end untranslated region, is known as pd5'26s.

Insertion of the structural protein gene cassette from pd5'26s into the pcDNA3 vector is performed as follows. Plasmid pd5'26s is digested with the enzyme Xba I and the 3'-recessed ends are made blunt by the addition of Klenow enzyme and dNTPs. The entire 4798 bp structural protein gene cassette is purified in a 1% agarose gel. Plasmid pcDNA3 is digested with the enzymes HindIII and Apa I and the ends are made blunt by the addition of T4 DNA polymerase enzyme and dNTPs, and the 5342 bp vector is purified in a 1% agarose gel. The two purified, blunt-end DNA fragments are subsequently ligated, and the resulting structural protein gene expression cassette vector is known as pCMV-d5'26s (see FIG. 11). Transfection of this DNA

into cells and selection for G418 resistance is performed as previously described.

Modifications of the CMV promoter/antisense-Sindbis structural protein vector also can be constructed using other viral, cellular, or insect-based promoters. Using common molecular biology techniques known in the art, the CMV promoter can be switched out of the Invitrogen pcDNA3 vector and replaced by promoters such as those listed previously. Other variation of this antisense packaging cassette may include, but are not limited to: the addition of 1 or more nucleotides between the first Sindbis nucleotide and the catalytic ribozyme, the use of longer or shorter HDV or other catalytic ribozyme sequences for transcript processing, the substitution of a precise transcription termination signal for the catalytic ribozyme sequence, or the antisense expression of structural protein gene cassettes using any downstream sequence recognized by an RNA polymerase which results in transcription of a structural protein gene mRNA.

Further, it should be noted that each of the vector-inducible constructs described contains sequences homologous to the Sindbis vector itself. Therefore, the potential exists for the generation of wild-type virus by recombination between the two RNA molecules. Additional modifications may be made to eliminate this possibility as described below.

#### 4. SEPARATION OF STRUCTURAL PROTEIN GENES TO PREVENT RECOMBINATION

Packaging cell lines may also be generated which segregate the integration and expression of the structural protein genes, allowing for their transcription as non-overlapping, independent RNA molecules. For example, the expression of capsid protein independently of glycoproteins E2 and E1, or each of the three proteins independent of each other, eliminates the possibility of recombination with vector RNA and subsequent generation of contaminating wild-type virus.

Specifically, capsid protein is expressed independently from an inducible expression vector, such that sequences which might result in recombination with vector RNA are eliminated. As an example, the capsid protein gene is amplified from plasmid pVGSP6GEN with a primer pair complementary to nucleotides 7632-7655 (forward primer) and 8415-8439 (reverse primer), with sequences as follows: Forward primer (Sin7632F)

5'-GTCAAGCTTGCTAGCTACAACACCACCACCA-  
TGAATAGAG-3' (SEQ. ID NO. 83)

Reverse primer (Sin8439R)

5'-CAGTCTCGAGTFACTACCACTCTTCTGTCCCT-  
TCCGGGGT-3' (SEQ. ID NO. 84)

In addition to their respective complementarities, the forward primer contains Nhe I and HindIII recognition sequences at its 5'-end, and the reverse primer contains both UAG and UAA translation stop codons and a Xho I recognition sequence at its 5'-end. Amplification is accomplished using a standard three-temperature cycling protocol, and the resulting amplicon is digested with the enzymes Nhe I and Xho I, and purified in a 1% agarose gel. Expression plasmid pMAM (Clontech), which contains a dexamethasone-inducible MMTV LTR promoter sequence, is digested with the enzymes Nhe I and Xho I and the plasmid DNA purified in a 1% agarose gel. The capsid protein gene fragment is ligated into the pMAM vector, and the resulting construct is known as pMAM/C. Plasmid pMAM/C is transfected into the appropriate cell line (for example BHK-21) as described previously and selection for stable transfectants is accomplished by using HAT (hypoxanthine, aminopterin, thymidine) media, supplemented with dialyzed fetal calf serum, mycophenolic acid and xanthine, as described by



Mulligan and Berg (PNAS 78:2072-2076, 1981). HAT-selected cell lines expressing capsid protein are identified following induction with  $10^{-6}$ M dexamethasone by lysing the cells with Lammeli sample buffer, separating the proteins using 12% SDS-PAGE, blotting onto nitrocellulose membrane, and detecting by western blot using polyclonal rabbit anti-Sindbis antibody. FIG. 21 shows expression of capsid protein in such cells, along with wild-type BHK-21 cells as a negative control, and Sindbis virus- infected BHK-21 cells as a positive control.

Alternatively, capsid protein is expressed using the lac-inducible vectors (Stratagene) described previously. The Sindbis capsid protein gene is amplified by PCR using primers Sin7632F and Sin8439R (described previously), and ligated with TA vector DNA (Stratagene). The resulting plasmid, designated TA/SinC, is digested with Eco RI, the termini are made blunt by the addition of Klenow fragment enzyme and dNTPs, and the capsid protein gene purified from a 1% agarose gel. Plasmid vectors pOP13 and pORSV1 are digested with Not I, their termini made blunt by the addition of Klenow fragment enzyme and dNTPs, and subsequently treated with calf intestinal alkaline phosphatase. The capsid protein gene is ligated with both pOP13 and pORSV1 vector DNA to generate the expression constructs designated pOP13CAP and pORSV1CAP, respectively. Each plasmid is co-transfected with p3'SS into the appropriate cell line as described previously, and selection for stable transfectants is accomplished using G418 and hygromycin selection. Cell lines expressing capsid protein are identified following IPTG induction by immunofluorescence using polyclonal rabbit anti-Sindbis antibody.

The glycoprotein genes, E1 and E2, are expressed together using one of the inducible systems previously described. For example, the Sindbis E1 and E2 genes are amplified from plasmid pVGSP6GEN using a primer pair complementary to Sindbis nucleotides 8440-8459 (forward primer) and Sindbis nts 11,384-11,364 (reverse primer). PCR amplification is performed using a standard three-temperature cycling protocol and the following oligonucleotide pair:

Reverse primer (11384R)

5'-TATATGCGGCCGCTCATCTTCGTGTGCTAGT-CAG-3' (SEQ. ID NO. 75)

Forward primer (8440F)

5'-TATATGCGGCCGCACCACTATGTCGCGAGCAC-CACTGGTCACG-3' (SEQ. ID NO. 85)

In addition to their respective complementarities, the forward primer contains an "in-frame" AUG translation initiation codon, and both primers contain a NotI recognition sequence at their 5'-ends. Following PCR amplification, the amplicon is digested with the NotI enzyme and purified in a 1% agarose gel. The resulting fragment is then ligated separately into the pOP13 and pOPRSV1 vectors (Stratagene), digested with Not I and treated with calf intestinal alkaline phosphatase, as described previously. These glycoprotein expression vectors are used to transfect cells that have been previously transfected with a capsid protein expression construct, and stable glycoprotein gene transfectants are identified by selection for G418 and hygromycin resistance.

Alternatively, the E1 and E2 glycoproteins are expressed under the control of the replicon-inducible junction region promoter, described previously. The ELVIS expression plasmid pVGELVISOSINBV-linker (Example 3) is digested with the enzyme Not I, and treated with calf intestinal alkaline phosphatase. PCR amplified Sindbis E1 and E2 glycoprotein genes digested with Not I (previous paragraph)

are then ligated to the ELVIS vector to generate a construct designated pVGELVIS-E1/E2. Plasmid pVGELVIS-E1/E2 subsequently is digested with the enzyme Bsp EI, removing most of the nonstructural protein gene coding region, and the remaining E1- and E2-containing vector DNA is re-ligated to itself, creating an inducible expression cassette identified as pVGELVd1-E1/E2. This glycoprotein expression vector is used to transfect cells that have been previously transfected with a capsid protein expression construct, and stable glycoprotein gene transfectants are identified by selection for G418 resistance. For both the capsid and envelope glycoprotein expression cassettes, additional mammalian or non-mammalian (including insect)-derived promoters, which may or may not be inducible, are readily substituted for those described above, using standard techniques known in the art.

#### 5. ASSEMBLING THE COMPONENTS TO CREATE THE ALPHAVIRUS PACKAGING CELL LINE

For example purposes, the BHK-21 cell line and replicon-inducible packaging expression cassette are used to demonstrate assembly of the components. However, other possible parent cell lines can be used to create alphavirus packaging cell lines and have been discussed previously. Briefly, BHK-21 cells (CCL 10) are grown at 37° C. in 5% CO<sub>2</sub> in Dulbecco's modified Eagle's Media (DMEM), 2 mM L-glutamine, and 10% fetal bovine serum (optimal media). Approximately  $5 \times 10^5$  BHK-21 cells, grown in a 35 mm petri dish, are transfected with 5 ug pLTR/SindIBspE using 5 ul of the Transfectam (Promega) cationic lipid reagent, in serum-free media conditions, as suggested by the supplier. However any method of transfection is rapidly substituted, i.e., by electroporation, calcium phosphate precipitation, or by using any of the readily available cationic liposome formulations and procedures commonly known in the art. At 24 hours post-transfection, the cells are trypsinized and reseeded in 100 mm dishes in 10 ml of optimal media, as described above, supplemented with 400 ug/ml of G418 (Gibco/BRL) and selected over a period of 5 to 7 days. Colonies displaying resistance to the G418 drug are then pooled, dilution cloned, and propagated. Individual clones are screened for high levels of Sindbis structural protein expression and functional packaging after transfection with Sindbis-luciferase vector RNA transcribed in vitro from SacI linearized plasmid pKSSINBV-luc (see Example 3). Specifically, clonally-derived pLTR/SindIBspE transfected BHK-21 cells (referred to as LTR/SindIBspE or BK-Bsp cells) grown in 60 mm petri dishes are transfected with 2 ug of Sindbis-luciferase vector RNA and overlaid with 3 ml of optimal media (see above). At 20 hours post-transfection, the supernatants are removed, and clarified by centrifugation for 30 min. at 3000 rpm in a Sorvall RT6000B tabletop centrifuge. In addition, the transfected cell monolayer is lysed in reporter lysis buffer (Promega) as described by the manufacturer, and assayed for luciferase expression as described previously.

The transfer of luciferase activity (and thus functional packaging) is tested by using 1 ml of the above supernatants to infect fresh monolayers of BHK-21 cells in 60 mm dishes. At 20 hours post-infection, the cell monolayers are lysed as described above and, tested for luciferase expression. As shown in FIG. 12, three clones (#13, 18, and 40) produce packaged Sindbis-luciferase vector and are the first examples of alphavirus packaging cell lines. In addition, transfected clone #18 cells are tested for increased vector packaging over a timecourse following transfection. Supernatants from transfected clone #18 cells are harvested at 20, 45, and 70 hours post-transfection, as described above, and



used to infect fresh monolayers of BHK-21 cells. FIG. 13 shows that Sindbis-luciferase vector packaging increases significantly at 45 hours post-transfection, as compared to 20 hours post-transfection. Expression also can be tested by western blot analysis using polyclonal rabbit anti-Sindbis antibodies (available in the literature).

#### C. INDUCIBLE VECTOR AND STRUCTURAL PROTEIN EXPRESSION FOR ALPHAVIRUS PRODUCER CELL LINES

##### 1. USE OF VIRAL PROMOTERS

The challenge of developing an alphavirus vector producer cell line lies in the question of whether a virus, whose infection of mammalian cells results almost exclusively in productive lytic cell death, can be modified to establish persistent infection in these same cells. One approach is to generate alphavirus vector producer lines from mosquito cells, where viral persistence often results after infection. However, the titer of infectious virus produced in persistently infected mosquito cells is only about  $1 \times 10^4$  PFU/ml, at least five orders of magnitude less than that observed after lytic infection of BHK cells by Sindbis.

Several strategies are described for inducible alphavirus vector producer cell lines, containing both vector and viral structural gene cassettes, such that productive cytolytic infection occurs only after the correct stimulus. Because these approaches operate on a "feed forward" level, any leakiness in the system will result in initiation of the alphavirus replication cycle and probable cell death. Therefore, tightly regulated control mechanisms are necessary for such a system.

The hallmark of development is the differentiation state-dependent pattern of gene expression. Briefly, gene expression patterns differ widely between undifferentiated and terminally differentiated states. Thus, a cell whose differentiation state can be controlled is likely an ideal host in which to derive an alphavirus vector producer cell line. In such a configuration, the vector expression cassette and, in some instances, structural components are coupled to terminal differentiation state-inducible promoters, according to the strategy described for ELVIS, and used to transform stably an undifferentiated host cell. Terminal differentiation of the host producer cell after induction with the appropriate stimuli coincidentally results in induction of the alphavirus replication cycle and production of packaged vector. Other strategies described herein, including antisense structural genes and heterologous viral expression systems, are readily coupled with cellular differentiation state-dependent promoters described below.

In this approach, four examples are described, using either a viral or cellular promoter which are active in only terminally differentiated cells.

It has been shown that mouse Polyomavirus (Py), SV40, and Moloney murine leukemia virus (M-MuLV), all are able to infect and enter undifferentiated mouse embryonal carcinoma (EC) cells, but the expression of their genes (and heterologous genes) and establishment of productive infection is blocked (Swartzendruber and Lehman, *J. Cell. Physiol.* 85:179-188, 1975; Peries et al., *J. Natl. Cancer Inst.* 59:463-465, 1977). These viral growth properties also have been demonstrated in two cell lines, PCC4 and F9, which are derived from the malignant stem cells of mouse teratocarcinomas. The block to viral propagation occurs at the level of transcription and replication, and maps to the enhancers, contained within the viral non-coding control regions (Linney et al., *Nature* 308:470-472, 1984; Fujimura et al., *Cell* 23:809-814, 1981; Katinka and Yaniv, *Cell* 20:393-399, 1980). When M-MuLV infects undifferentiated

EC cells, the viral DNA integrates into the genome. However, as stated above, expression of viral genes or of heterologous genes is blocked. This block of viral expression is released upon terminal differentiation of EC cells by addition of retinoic acid to the growth medium.

To test the RNA expression properties of the pVGELVIS construct in EC cells, plasmid DNA is complexed with LIPOFECTAMINE (GIBCO-BRL, Gaithersburg, Md.) according to the conditions suggested by the supplier (ca. 5 g DNA/8 g lipid reagent) and added to 35 mm wells containing undifferentiated PCC4 or F9 cells (Fujimura et al., 1981, *Cell* 23:809-814) at approximately 75% confluency. The development of cytopathic effects (CPE), and the level of Sindbis productive infection, quantitated by plaque assay of media supernatant, is determined at regular intervals over 5 days in undifferentiated and differentiated transfected PCC4 or F9 cells. Differentiation of F9 and PCC4 cells is accomplished by addition of retinoic acid (Sigma Chemical Co., St. Louis, Mo.), at a final concentration of 1M.

It has been proposed that the hierarchy of relative expression of heterologous genes observed in undifferentiated EC cells infected with M-MuLV vectors may be in part insertional dependent (Linney et al., 1987, *J. Virol.* 61:3248-3253). Thus, undifferentiated EC cells transfected with pVGELVIS may likely produce different results, in terms of transcription of the Sindbis genomic cDNA and, in turn, initiation of the viral life cycle. In this event, following G418 selection of pVGELVIS transfected undifferentiated EC cells, remaining cells are cloned and expanded. The cell clones are then tested for the production of Sindbis virus after differentiation by addition of retinoic acid (Sigma Chemical Co., St. Louis, Mo.), at a final concentration of 1M.

To isolate vector packaging cell lines, whose production of structural proteins in the presence of Sindbis NSP is cell differentiation state dependent, undifferentiated F9 or PCC4 cells are transfected with pLTR/SINdIBspE and G418 selected as described above. Differentiation state-sensitive clones are then selected by infection at high multiplicity with packaged SIN-luc vector. Clones which are resistant to cell lysis or do not produce packaged SIN-luc vector particles, are candidate vector packaging clones. These candidate clones are tested for SIN-luc vector particle production following terminal differentiation with retinoic acid, as described.

The murine wild type polyomavirus (Py) is unable to replicate in the teratocarcinoma cell lines PCC4 or F9. This block of replication in undifferentiated cells occurs at the level of transcription of early region (i.e., T antigen) genes, and is released by induction of terminal differentiation with vitamin A. Py mutants which are able to establish productive infection in undifferentiated PCC4 and F9 cells map to the viral enhancer region. The genesis of an embryonic tissue specific transcriptional enhancer element has resulted in these mutants. In order to exploit this property of inhibition of Py replication in undifferentiated teratocarcinoma cell lines, the viral regulatory non-coding region, including the enhancer, is coupled to the genomic cDNA of Sindbis virus, according to the ELVIS strategy. The precise transcriptional start site of the Py early region has been determined (see Tooze, *DNA Tumor Viruses*). The PCC4 and F9 cell lines are stably transformed with the Py-Sindbis vectors. In this model Sindbis productive infection occurs after addition of retinoic acid to the culture medium and induction of terminal differentiation.

The Py non-coding region from bases 5021-152, which includes the sequences corresponding to the viral enhancers,

21 bp repeats, replication origin, CAAT and TATA boxes, and the early mRNA transcription 5' cap site, is positioned at the 5' viral end such that *n vivo*, only a single capped C residue is added to the Sindbis 5' end. Juxtaposition of the Py non-coding region and the Sindbis 5' end is accomplished by overlapping PCR as described in the following detail. Amplification of the Py non-coding region in the first primary PCR reaction is accomplished in a reaction containing the pBR322/Py, strain A2 plasmid (ATCC number 45017-p53.A6.6(pPy-1)) and the following primer pair: Forward primer: Pybgl5021F (buffer sequence/Bgl II recognition sequence/Py nts 5021-5043)

5'-TATATAGATCTCTTGATCAGCCTTCAGAAGATGGC (SEQ. ID NO. 86)

Reverse primer: SINPy152R (SIN nts 5-1/Py nts 152-134)

5'-TCAATGGCGGGAAGAGGCGGTTGG (SEQ. ID NO. 87)

PCR amplification of the Py non-coding region with the primer pair shown above is performed using the Thermalase thermostable DNA polymerase (Amersco Inc., Solon, Ohio) and the buffer containing 1.5 mM MgCl<sub>2</sub>, provided by the supplier. Additionally, the reaction contains 5% DMSO, and the Hot Start Wax beads (Perkin-Elmer), using the following PCR amplification protocol shown below:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	0.5	
72	10	1

Amplification of the Sindbis 5' end in the second primary PCR reaction is accomplished in reaction containing the pVGSP6GEN clone and the following primer pair: Forward primer: (Py nts 138-152/SIN nts 1-16)

5'-CCGCCTCTTCCCGCCATTGACGGCGTAGTAC (SEQ. ID NO. 88)

Reverse primer: (SIN nts 3182-3160)

5'-CTGGCAACCGGTAAGTACGATAC (SEQ. ID NO. 18)

PCR amplification of Sindbis 5' end region with the primer pair shown above in with the reaction conditions described above, using the following PCR amplification protocol shown below:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	3.0	
72	10	1

The 442 bp and 3202 bp products from the primary PCR reactions are purified with GENECLEAN (BIO 101), and used together in a PCR reaction with the following primer pair:

Forward primer: Pybgl5021F (buffer sequence/Bgl II recognition sequence/Py nts 5021-5043):

5'-TATATAGATCTCTTGATCAGCCTTCAGAAGATGGC (SEQ. ID NO. 89)

Reverse primer: (SIN nts 2300-2278):

5'-GGTAACAAGATCTCGTGCCGTG (SEQ. ID NO. 19)

PCR amplification of the of the primer PCR amplicon products with the primer pair shown above is with the reaction conditions described above, using the following PCR amplification protocol shown below:

Temperature (°C.)	Time (Min.)	No. Cycles
94	2	1
94	0.5	
55	0.5	35
72	3.0	
72	10	1

The 20 3' terminal bases of the first primary PCR amplicon product overlaps with the 20 5' terminal bases of the second primary PCR amplicon product; the resultant 2,742 bp overlapping secondary PCR amplicon product is purified by 0.8% agarose/TBE electrophoresis, digested with Bgl II, and the 2,734 bp product is ligated into pcDNASINbgl/xbal (see Example 3) treated with Bgl II and CIAP. The resulting construction is 16,641 bps and is known as ELVIS-PySIN. In order to construct a structural protein expression vector similar to pLTR/SINdIBspE for the derivation of vector packaging cell lines, the ELVIS-PySIN construction is digested to completion with Bsp EI, and religated under dilute conditions, in order to accomplish deletion of the nonstructural proteins between bases 422-7054. This construction is known as ELVIS-PySINdIBspE.

ELVIS-PySIN plasmid DNA is complexed with LIPO-FECTAMINE (GIBCO-BRL, Gaithersburg, Md) according to the conditions suggested by the supplier (ca. 5 g DNA/8 g lipid reagent) and added to 35 mm wells containing undifferentiated PCC4 or F9 cells at approximately 75% confluency. The development of cytopathic effects (CPE), and the level of Sindbis productive infection, quantitated by plaque assay of media supernatant, is determined at regular intervals of 5 days in undifferentiated and differentiated PCC4 or F9 cells. Differentiation of F9 and PCC4 cells is accomplished by addition of retinoic acid (Sigma Chemical Co., St. Louis, Mo.), at a final concentration of 1 mM.

If the undifferentiated EC cells demonstrate a heterologous response to transfection with ELVIS-PySIN, remaining cells not lysed by Sindbis virus propagation following G418 selection of pVGELVIS transfected undifferentiated EC cells are cloned and expanded. The cell clones are then tested for the production of Sindbis virus after differentiation, by addition of retinoic acid (Sigma Chemical Co., St. Louis, Mo.), at a final concentration of 1 mM.

Isolation of vector packaging cell lines stably transfected with ELVIS-PySINdIBspE, having a cell differentiation state dependent pattern of expression of structural proteins in the presence of Sindbis NSP, is accomplished as described above for the pLTR/SINdIBspE plasmid.

In order to demonstrate the feasibility of an inducible Sindbis vector producer cell line, the reporter gene expression from the ELVIS-luc vector, whose construction is described in Example 3, section E, after transfection of BHK and undifferentiated F9 cells is determined. In addition, both cell types are infected with packaged SIN-luc vector, whose production is described in Example 3 section C. This later experimental group serves as a control that expression restriction (if any) lies at the level of transcription rather than a receptor difference on unique cell types. The results of this study, shown in FIG. 14, demonstrate that the expression of luciferase is inhibited in undifferentiated F9 cells. The level of luciferase expression in BHK cells transfected with ELVIS-luc and BHK and undifferentiated F9 cells infected with packaged SIN-luc vector is similar. Thus, in ELVIS-luc

transfected undifferentiated F9 cells, transcription from the LTR and subsequent luciferase expression via the Sindbis vector autocatalytic pathway is inhibited. This study demonstrates that packaging cell lines can be developed where synthesis of Sindbis vector or Sindbis vector packaging is inducible and controlled by the differentiation state of the cell.

## 2. USE OF CELLULAR PROMOTERS.

The third example of this strategy uses the  $\beta$ -globin locus control region. The  $\beta$ -globin multigene cluster contains five developmentally regulated genes. In the early stages of human development, the embryonic yolk sac is the hematopoietic tissue and expresses the  $\epsilon$ -globin gene. This is followed by a switch to the  $\gamma$ -globin gene in the fetal liver and the  $\delta$ - and  $\beta$ -globin genes in adult bone marrow (Collins and Weissman, 1984, *Prog. Nucleic Acid Res. Mol. Biol.* 31:315).

At least two mouse erythroleukemia lines, MEL and Friend, serve as models for terminal differentiation dependent expression of  $\beta$ -globin. Expression of  $\beta$ -globin is observed in these lines only after induction of terminal differentiation by addition of 2% DMSO to the growth medium.

The entire  $\beta$ -globin locus is regulated by the locus control region (LCR). Within the LCR is the dominant control region (DCR) residing within the DNase I hypersensitive region, which is 5' of the coding region. The DCR contains five DNase I hypersensitive (HS1- HS5) sites. The DCR directs high level site of integration independent, copy number dependent expression on a linked human  $\beta$ -globin gene in transgenic mice and stably transfected mouse erythroleukemia (MEL) cells (Grosveld et al., 1993, *CSHSQB* 58:7-12). In a recent study (Ellis et al., 1993, *EMBO* 12:127-134), concatamers of a synthetic core coinciding to sequences within HS2 were shown to function as a locus control region.

In order to accomplish the differentiation state dependent expression of alphavirus vectors, the viral genomic cDNA is juxtaposed with a promoter containing a tandem synthetic core corresponding to the LCR HS2 site. Alternatively, the desired alphavirus vector construct can be inserted downstream of the LCR in the endogenous-globin gene by homologous recombination. In such a strategy, the  $\beta$ -globin transcription initiation site after terminal differentiation would be first determined, in order that the alphavirus vector could be placed precisely at the start site.

Initiation of a lytic viral life cycle is controlled by the differentiation state of the host cell is applicable to other systems, where the control of viral induced cytopathology is desired.

Yet another approach to regulating alphavirus gene expression through a differentiation state sensitive promoter is the use of the retinoic acid receptor (RARA) and acute promyelomonocytic leukemia cells (APL). APL cells are clonal myeloid precursors characterized by high growth rate and differentiation arrest. A non-random chromosomal translocation breakpoint, t(15;17)(q22;21), occurs in almost all patients with APL. The RARA gene has been localized to chromosome 17q21. Analysis of APL mRNA from patients has shown that most APL breakpoints occur within the second intron of the RARA gene and result in abnormal fusion transcripts. Co-transfection assays with RARA and PML-RARA fusion cDNAs have demonstrated that the resulting fusion proteins can antagonize wild-type RARA in the presence of retinoic acid. These studies implicate PML-RARA fusion protein in the molecular pathogenesis of APL. Importantly, a significant number of patients achieve com-

plete remission after all-trans retinoic acid treatment (ATRA). High concentration of ATRA may overcome the RARA deficiency leading to high levels of RA in the nucleus. Differentiation of the APL cells can then be achieved through activation of RARA responsive genes. RA can induce differentiation of a number of cell lines, including the human leukemia line HL-60.

The retinoic acid receptor is a member of a nuclear receptor superfamily that includes the thyroid and steroid hormone receptors. Four different forms of the human RAR have been identified, and the corresponding cDNAs cloned and characterized. In order to accomplish the differentiation state dependent expression of Sindbis vectors, viral genomic cDNA is juxtaposed with the RARA DNA binding site, creating ELVIS-RARASIN. As with the strategy proposed for ELVIS-PySIN expression in undifferentiated EC cells, differentiation sensitive ELVIS-RARASIN expressing cells are isolated.

## 3. INSERTION OF VECTOR CONSTRUCTS INTO DIFFERENTIATION STATE CONTROLLED INDUCIBLE PROMOTERS

Generation of clones whose expression of heterologous genes from Sindbis vectors positioned in the ELVIS configuration as described in Example 3 is differentiation state dependent, is accomplished as described above for the pVGELVIS, pLTR/SindBspE plasmids. Generation of clones whose production of vector particles is differentiation state dependent, is accomplished by transfecting the isolated differentiation dependent vector packaging clones described above with ELVIS heterologous gene expression vectors. Clones having the desired phenotype or vector production after retinoic acid induced differentiation are isolated as described above.

## D. STRUCTURAL PROTEIN EXPRESSION FROM A HETEROLOGOUS ASTROVIRUS JUNCTION REGION

Among the critical properties of a vector packaging system are a cell line which expresses the structural components necessary to generate an infectious particle, without the creation of wild-type virus through recombination between vector and structural gene components. These two desired properties of the packaging cell line are accomplished in the retrovirus based systems through the constitutive expression of the gag/pol and env genes on individual heterologous RNA polymerase II expression cassettes.

Another important aspect of vector packaging cell lines is to derive a system which mimics as closely as possible the normal replication strategy of the wild type virus. This issue is important in terms of the observed titer level of packaged recombinant vector. Synthesis of the viral structural proteins during alphavirus infection is accomplished after transcription of high levels of subgenomic mRNA from the junction region promoter, followed by efficient translation into the structural proteins. The junction region promoter is functional only in the antisense orientation and synthesis of the antigenomic RNA occurs after translation of the nonstructural proteins, thus delaying the expression of the structural proteins. It follows that, with regard to alphavirus, it would be desirable to construct a packaging cell line in which synthesis of the structural proteins is initiated from the junction region promoter, which in turn is activated by nonstructural proteins expressed from the recombinant vector molecule.

It is known that a relatively high frequency of recombination occurs between RNA genomic molecules occurs during infection with Sindbis virus via a copy choice mechanism (*PNAS* 88:3253-3257, 1991). Recombination between vector and junction region/structural gene cassettes would

result in the generation of wild-type Sindbis virus, perhaps at a level of 1 wild-type virus per million of packaged vector particles (*Liljestrom Bio Technology* 9:1356-1361, 1991). One way to mitigate the generation of wild-type virus is to separate the structural genes onto separate expression cassettes, an approach which has been discussed previously in Example 7.

An additional approach to diminish the level of wild-type virus production in alphavirus vector packaging cell lines is to express the structural proteins under the control of Astrovirus genetic elements. A schematic for this configuration is depicted in FIG. 15. Similar to alphaviruses, the expression of Astrovirus structural proteins incorporates a junction region strategy, in which high levels of structural proteins are synthesized from a subgenomic message. The Astrovirus expression cassette may consist of one of the two following ordered elements: (1) inducible promoter/Astrovirus 5' end/Astrovirus junction region/alphavirus structural gene/Astrovirus 3' end, or (2) antisense Astrovirus 3' end/antisense alphavirus structural gene/antisense Astrovirus junction region/antisense Astrovirus 5' end/Hepatitis Delta virus ribozyme, or other configurations described in Example 7. In both configurations, the expression unit is amplified by the Astrovirus nonstructural proteins through the same mechanism that occurs during viral replication. Since multiple rounds of subgenomic mRNA synthesis initiated from the junction region occur from each expression unit, amplification of the expression unit by the Astrovirus nonstructural proteins results in the production of very high levels of alphavirus structural proteins. The second configuration of the alphavirus structural protein expression cassette described above may function better than the first, because the primary transcript of the toxic alphavirus structural gene is antisense. Although expression of the structural genes in the first configuration should not occur until synthesis of the negative strand followed by synthesis of the positive subgenomic RNA from the junction region, the antisense nature of the primary transcript in the second configuration represents an additional level of control to prevent cytotoxic protein expression.

It is likely that no wild-type virus would be generated in a packaging cell line in which the alphavirus virus structural proteins are synthesized individually from Astrovirus junction region expression cassettes. Recombination between the nonstructural protein region of the vector and an Astrovirus structural protein expression cassette would result in a molecule in which Astrovirus cis elements were coupled with alphavirus genes, a nonviable combination. Correct coupling of alphavirus cis and trans elements would require two precise recombination events between the vector and the Astrovirus expression cassette, between the Astrovirus junction region and structural gene ATG, and between the structural gene termination codon and the Astrovirus 3' end. In order to generate wild type virus, this dual recombination event would have to occur three times on the same molecule (six total events), to incorporate the three separated alphavirus structural genes.

In order to diminish any possible toxicity of the Astrovirus proteins, synthesis of the Astrovirus expression cassettes may also be controlled by inducible promoters. One possibility is to use the lac operon, according to the "lac-switch" system described previously in Example 7 (Stratagene). The constitutive level of expression of the lac operon controlled gene in the absence of the gratuitous inducer IPTG is about 10 copies of RNA per cell. The inducible promoter corresponding to the Astrovirus/alphavirus structural gene expression cassette may be the lac operon or other suitable

promoters which have very low level of constitutive expression. Construction of packaging cell lines of these configurations, in which the control of alphavirus proteins is directed by a heterologous virus should result in the generation of high titer wild-type virus free packaged vector particles.

#### Example 8

#### ALTERNATIVE VIRAL VECTOR PACKAGING TECHNIQUES

Various alternative systems can be used to produce recombinant alphavirus particles carrying the vector construct. Each of these systems takes advantage of the fact that baculovirus, and the mammalian viruses vaccinia and adenovirus, among others, have been adapted recently to make large amounts of any given protein for which the gene has been cloned. (Smith et al., *Mol. Cell. Biol.* 3:12, 1983; Piccini et al., *Meth. Enzymology* 153:545, 1987; and Mansour et al., *Proc. Natl. Acad. Sci. USA* 82:1359, 1985). These and other viral vectors are used to produce proteins in tissue culture cells by insertion of appropriate genes into the viral vector and can be readily adapted to make alphavirus vector particles.

For example, adenovirus vectors are derived from nuclear replicating viruses and can be modified so they are defective. Heterologous genes are inserted into these vectors either by in vitro construction (Ballay et al., *EMBO J.* 4:3861, 1985) or by recombination in cells (Thummel et al., *J. Mol. Appl. Genetics* 1:435, 1982), and used to express proteins in mammalian cells. One preferred method is to construct plasmids using the adenovirus major late promoter (MLP) driving: (1) alphavirus structural proteins; and (2) an alphavirus vector construct. The alphavirus vector in this configuration still contains a modified junction region, and would allow the transcribed RNA vector to be self-replicating, as in previously described configurations.

These plasmids are then used to make adenovirus genomes in vitro (Ballay et al., *EMBO J.* 4:3861, 1985). The recombinant adenoviral genomes, which are replication defective, are separately transfected into 293 cells (ATCC #CRL 1573, a human cell line making adenovirus E1A protein), to yield pure stocks of defective adenovirus vectors expressing either alphavirus structural proteins or alphavirus vectors. Since the titres of such vectors are typically  $10^7$ - $10^{11}$ /ml, these stocks are then used to infect tissue culture cells simultaneously at high multiplicity of infection, resulting in the production of alphavirus proteins and vector genomes at high levels. Since the adenovirus vectors are defective, little or no direct cell lysis will occur and vectors are harvested from the cell supernatants. Similar approaches are readily carried out using recombinant vaccinia virus vectors constructed by inserting the alphavirus sequences into the shuttle plasmid pK (Bergmann et al., *Eur. J. Immunol.* 23:2777, 1993) for in vivo recombination into the vaccinia WR strain.

Other viral vectors, such as those derived from unrelated vectors (e.g., RSV, MMTV or HIV), also may be used in the same manner to generate packaged vectors from primary cells. In one embodiment, these adenoviral vectors are used in conjunction with primary cells, giving rise to recombinant alphavirus particles.

An alternative expression system also has been described in which chimeric HIV/poliiovirus genomes result in the generation of chimeric minireplicons (*J. Virol.* 65:2875, 1991) capable of expressing fusion proteins. These chimeric

poliovirus minireplicons, in which HIV-1 gag-pol sequences were substituted for the VP2 and VP3 capsid genes of the P1 capsid of poliovirus, were later demonstrated to be encapsidated and produce infectious particles by using a recombinant vaccinia virus (VV-P1) that expresses the substituted poliovirus capsid precursor P1 proteins defective in the chimeric minireplicon (*J. Virol.* 67:3712, 1993). For use in accordance with this invention, the alphavirus vector genome is substituted for the P1 capsid sequences and used as a means for providing polio-pseudotyped alphavirus vectors after transfecting in vitro transcribed alphavirus vector RNA transcripts into the cell line. Conversely, alphavirus structural proteins also may be substituted for the VP2 and VP3 proteins, subsequently providing an alternative packaging cell line system for alphavirus based vectors.

In an alternative system, several components are used, including: (1) alphavirus structural proteins made in the baculovirus system using techniques described by Smith et al. (*supra*) (or in other protein production systems, such as yeast or *E. coli*); (2) viral vector RNA made in the known T7, SP6 or other in vitro RNA-generating system (Flamant et al., *J. Virol.* 62:1827, 1988); (3) tRNA transcribed in vitro or purified from yeast or mammalian tissue culture cells; (4) liposomes (with embedded envelope glycoproteins); and (5) cell extract or purified necessary components when identified (typically from mouse cells) to provide RNA processing, and any or other necessary cell-derived functions.

Within this procedure, components (1), (2) and (3), from above, are mixed, and then envelope glycoprotein associated alphavirus proteins, cell extract and pre-liposome mix (lipid in a suitable solvent) are added. In an alteration of the procedure, the alphavirus envelope glycoproteins are embedded in the liposomes prior to addition to the mixture of (1), (2), and (3). The resulting mixture is then treated (e.g., by sonication, temperature manipulation, or rotary dialysis) to allow envelopment of the viral nucleocapsid particles with lipid plus embedded alphavirus envelope glycoprotein in a manner similar to that for liposome encapsidation of pharmaceuticals (Gould-Fogerite et al., *Anal. Biochem.* 148:15, 1985). This or similar procedures can be used to produce high titres of packaged alphavirus vectors without the requirement of establishing intermediate packaging cell lines.

#### Example 9

##### CELL LINE OR ISSUE SPECIFIC ALPHAVIRUS VECTORS-"HYBRID ENVELOPES"

The tissue and cell-type specificity of alphaviruses is determined primarily by the virus-encoded envelope proteins, E1 and E2. These virion structural proteins are transmembrane glycoproteins embedded in a host cell-derived lipid envelope that is obtained when the viral particle buds from the surface of the infected cell. The envelope surrounds an icosahedral nucleocapsid, comprised of genomic RNA complexed with multiple, highly ordered copies of a single capsid protein. The E1 and E2 envelope glycoproteins are complexed as heterodimers which have been reported to assemble into trimeric structures, forming the characteristic "spikes" on the virion surface. In addition, the cytoplasmic tails of these proteins interact with the nucleocapsids, initiating the assembly of new viral particles (*Virology* 193:424, 1993). Properties ascribed to the individual glycoproteins of Sindbis virus include receptor binding by glycoprotein E2 (*Virology* 181:694, 1991) and glycoprotein E1-mediated fusion of the virion envelope and the

endosomal membrane, resulting in delivery of the nucleocapsid particle into the cytoplasm (*New Aspects of Positive-Stranded RNA Virus*, pp. 166-172, 1990).

The present invention recognizes that by disrupting glycoprotein activity (in particular, but not limited to that of E2) and co-expressing an intact heterologous glycoprotein, or by creating hybrid envelope gene products (i.e., specifically, an alphavirus envelope glycoprotein having its natural cytoplasmic domain and membrane-spanning domain, with its exogenous binding domain replaced by the corresponding domain(s) from a different envelope glycoprotein, or by replacing the E2 and/or E1 glycoproteins with those of other alphaviruses or their derivatives which differ from that of the vector in their tissue tropism, the host range specificity may be altered without disrupting the cytoplasmic functions required for virion assembly. Alternatively, by replacing one or more of the alphavirus structural proteins with the structural protein(s) of another virus and introducing the corresponding viral packaging sequence into the alphavirus vector construct, assembly of recombinant alphavirus vector constructs into particles of other virus types can be achieved. Thus, recombinant alphavirus particles can be produced which have an increased affinity for pre-selected target cells, depending on the tropism of the protein molecule(s) or domain(s) introduced.

In one embodiment, substitution of the analogous envelope glycoproteins E1 and/or E2 from other alphaviruses or their variants is performed to alter tissue tropism. For example, Venezuelan equine encephalitis virus (VEE) is an alphavirus which exhibits tropism for cells of lymphoid origin, unlike its Sindbis virus counterpart. Therefore, Sindbis-derived vector constructs packaged in a cell line expressing the VEE structural proteins display the same lymphotropic properties as the parental VEE virus from which the packaging cell structural protein gene cassette was obtained.

Specifically, the Trinidad donkey strain of VEE virus (ATCC #VR-69) is propagated in BHK-21 cells, and virion RNA is extracted using procedures similar to those described in Example 1. The entire structural protein coding region is amplified with a primer pair whose 5'-ends map, respectively, to the authentic AUG translational start site, including the surrounding Kozak consensus sequence, and UGA translational stop site. The forward primer is complementary to VEE nucleotides 7553-7579, and the reverse primer is complementary to VEE nucleotides 11206-11186 (sequence from Kinney et al., *Virology* 170:19-30, 1989). PCR amplification of VEE cDNA corresponding to the structural protein genes is accomplished using a two-step reverse transcriptase-PCR protocol as described above, the VEE genome RNA as template, and the following oligonucleotide pair:

Forward primer (VEE 7553F)

5'-TATATATATGCGGCCGCACCGCCAAGATGTTCCGTTCCAGCCA-3' (SEQ. ID NO. 90)

Reverse primer (VEE 11206R)

5'-TATATATATGCGGCCGCTCAATTATGTTTCTGTGGT-3' (SEQ. ID NO. 91)

In addition to their respective complementarities to the indicated VEE nucleotides, each primer includes a Not I recognition sequence at their 5' ends. Following PCR amplification, the 3800 bp fragment is purified in a 1% agarose gel and digested with the enzyme Not I. The resulting fragment is then ligated separately into the pOP13 and pOPRSV1 vectors (Stratagene) described previously, which are digested with Not I and treated with calf intestinal

alkaline phosphatase. These resulting vectors, which contain the entire VEE structural protein coding sequence, are known as pOP13-VEESP and pOPRSV1-VEESP. The use of these clones in the development of VEE-based packaging cell lines follows that described for Sindbis packaging lines. Alternatively, the PCR amplified VEE structural protein gene fragment digested with *NotI* is ligated into the replicon inducible ELVIS cassette described in Example 7. Plasmid pVGELVISBV-linker is digested with *Bsp* EI to remove most nonstructural protein coding sequences, and the vector is then re-ligated with itself to generate the construct pVGELVISdl-linker. Subsequently, this plasmid is digested with *NotI*, treated with calf intestinal alkaline phosphatase, and ligated with the *NotI* digested VEE fragment to generate the expression cassette pVGELVdIVEE. Plasmid DNA of this construct is transfected into the appropriate cell line and selection for G418 resistance is performed as described in Example 7. In addition, variations of the vector-inducible or lac operon-VEE structural protein gene expression vectors may be constructed using other systems described herein. Additionally, other variations may be constructed which combine the capsid protein gene of one alphavirus (for example, Sindbis) with the envelope glycoprotein genes of another alphavirus (for example, VEE) in a split gene approach, as described in Example 7. Furthermore, variants of VEE, and other alphaviruses and their variants differing in tissue tropism, are useful when following this approach.

In another embodiment, a RNA packaging signal derived from another virus is inserted into the alphavirus vector to allow packaging by the structural proteins of that corresponding virus. For example, the 137 nt. packaging signal from hepatitis B virus, located between nts. 3134 and 88 and spanning the precore/core junction (Junker-Niepmann et al. *EMBO J.* 9:3389, 1990), is amplified from an HBV template using two oligonucleotide primers. PCR is performed using a standard three temperature cycling protocol, plasmid pHBV1.1 (Junker-Niepmann et al. *EMBO J.* 9:3389, 1990) as the template, and the following oligonucleotide pair, each of which contain 20 nucleotides complementary to the HBV sequence and flanking *Apal* recognition sequences:

Forward primer (HBVpkgF)  
5'-TATATGGGCCCTACATGTCCCACTGTTCAAG-3'  
(SEQ. ID NO. 117)

Reverse primer (HBVpkgR)

5'-TATATGGGCCCGTACGGAAGGAAAGAAGTCA-3'  
(SEQ. ID NO. 118) Following amplification, the PCR amplicon is digested with *Apal* and purified from a 1.5% agarose gel using MERMAID™ (Bio101). Sindbis vector plasmid pKSSINdJRSjrc (Example 3) also is digested with *Apal*, under limited conditions to cleave at only one of its two sites, followed by treatment with CIAP, purification from a 1% agarose gel, and ligation with the above-synthesized HBV amplicon, to produce a construct designated pKSSINhvbJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Cell lines which express the HBV core, preS/S, and P proteins necessary for packaging of the RNA sequence are derived by modification of helper plasmid pCH3143 (Junker-Niepmann et al., *EMBO J.* 9:3389, 1990) to include a selectable marker. An expression cassette containing the neomycin resistance marker is obtained by digestion of plasmid pBK-RSV (Stratagene) with *Mst* II and blunt-ending with Klenow fragment. The selectable marker is then ligated into any one of several unique sites within pCH3143 that have been digested and their termini made blunt. The resulting construct is transfected into a desired cell line, for

example, mouse hepatoma line Hepa1-6 (ATCC #CRL1830), and selected using the drug G418, as described in Example 7. Introduction of the pKSSINhvbJR vector, or related RNA- or DNA-based alphavirus vectors, results in the production of packaged vector particles with the same hepatotropism as HBV.

Similarly, the packaging signal from a coronavirus can be incorporated into the alphavirus vector. For example, the 190 nt packaging signal from mouse hepatitis virus (MHV), comprising nts 2899 to 3089 (Fosmire et al., *J. Virol.* 66:3522, 1992), is amplified in a standard three cycle PCR protocol using THERMALASE™ polymerase, DlsSF plasmid MP51-2 (Fosmire et al., *J. Virol.* 66:3522, 1992) as the template, and the following oligonucleotides, which contain flanking *Apal* recognition sites:

Forward primer (MHVpkgF)

5'-TATATGGGCCCATTTTGGTTTTGCTATGCGTAA-3'  
(SEQ. ID NO. 119)

Reverse primer (MHVpkgR)

5'-TATATGGGCCCATCGAGGTGAGAAAGAGGAC-3'  
(SEQ. ID NO. 125) Following amplification, the PCR amplicon is digested with *Apal*, purified from a 1.5% agarose gel using MERMAID™, and ligated into pKSSINdJRSjrc, prepared as described for HBV. The resulting construct is designated pKSSINmhvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. Packaging of vectors modified with this MHV sequence is accomplished by using expression cassettes which produce each of the required coronavirus structural proteins: nucleocapsid (N protein; Armstrong et al., *NAR* 11:883, 1983); membrane (M protein; Armstrong et al., *Nature* 308:751, 1984); and spike (S protein; Luytjes et al., *Virology* 161:479, 1987). Preferably, these proteins are inserted into the vector-inducible pVGELVdI-linker plasmid (described previously in this example) and selected for with the G418 drug following transfection into the appropriate cell type. Other expression methodologies (see Example 7) may also be readily utilized. Additional coronaviruses, for example, human coronaviruses OC43 (ATCC #VR-759) and 229E (ATCC #VR-740), can be readily used in place of MHV to produce packaged recombinant alphavirus particles which show tropism for cells in the respiratory tract.

Similarly, the packaging signal from a retrovirus can be incorporated into an alphavirus vector construct. For example, the 351 nt extended packaging signal ( $\Psi$ +) from Mo-MLV, corresponding to nts 212 to 563 (Mann et al., *Cell* 33:153, 1983), is amplified in a standard three cycle PCR protocol as described above, using plasmid pMLV-K (Miller, *J. Virol.* 49:214, 1984) as template and the following oligonucleotides, each of which contain a flanking *Apal* recognition site:

Forward primer (MLVpkgF)

5'-TATATGGGCCCTGTATCTGGCGGACCCGTGG-3'  
(SEQ. ID NO. 126)

Reverse primer (MLVpkgR)

5'-TATATGGGCCCGCAGACAAGACGCGCGGCGC-3'  
(SEQ. ID NO. 127)

Following amplification, the PCR amplicon is digested with *Apal*, purified from a 1.5% agarose gel using GENECLEAN™, and ligated into plasmid pKSSINdJRSjrc, prepared as described above. The resulting construct is designated pKSSINmlvJR. Other alphavirus vectors (see Example 3) are readily modified in a similar manner. The generation of a retroviral-derived producer cell line for packaging and production of the above alphavirus

vector constructs is accomplished by transfecting an appropriate packaging cell line, for example amphotropic line DA (WO 92/05266), and selecting for resistance to the drug G418, as described previously.

In each case, the packaging sequences from HBV, 5 coronavirus, retrovirus, or any other virus, also may be incorporated into alphavirus vectors at locations other than those outlined above, provided the location is not present in the subgenomic transcript. For example, the next most preferable site of insertion is the carboxy-terminal region of nonstructural protein 3, which has been shown to be highly variable in both length and sequence among all alphaviruses for which sequence information is available. Further, these applications are not limited by the ability to derive the corresponding packaging cell lines, as the necessary structural proteins also may be expressed using any of the alternative approaches described in Example 8. 10

In yet another embodiment, a heterologous glycoprotein or cellular ligand is expressed in the lipid bilayer of a packaging cell line for producing enveloped recombinant alphavirus particles. This approach is similar to that described in Example 8 for the production of VSV-G pseudo-typed alphavirus vectors, except that in this configuration, the E2 receptor-binding function is inactivated by insertion, deletion, or site-specific mutagenesis. As an example, receptor binding function of E2 can be inactivated by techniques known in the art to restrict vector particle tropism to that which is supplied by the heterologous glycoprotein or cellular ligand. In addition to the example of VSV-G pseudo-typing, other viral glycoproteins which target specific cellular receptors (such as the retroviral HIV gp120 protein for CD4 cell targeting) are utilized when expressed from standard vectors stably transfected into alphavirus packaging cell lines. 15

In another configuration, chimeric glycoproteins are prepared which allow for targeting of alphavirus vector constructs into particular cell lines in vitro or tissue types in vivo. To construct such a chimeric glycoprotein, specific oligonucleotides encoding the ligand binding domain of the desired receptor, plus homologous alphavirus sequences (which include a unique specific restriction endonuclease site), are used to amplify an insert sequence that can be substituted into an alphavirus structural protein expression cassette. Alternatively, limited Bal-31 digestions from a convenient restriction enzyme site are performed in order to digest back to a permissive insertion site, followed by blunt end ligation of a fragment encoding a small receptor binding domain, an entire viral glycoprotein, or cell surface ligand. As an example, peptides corresponding to the principal neutralizing domain of the HIV gp120 envelope protein (Virology 185:820, 1991) can be used to disrupt normal E2 tropism and provide CD4 cell targeting. 20

While inclusion of the HIV gp120 neutralizing domain illustrates one example of a hybrid or chimeric envelope protein, the possibilities are not limited to viral glycoproteins. For example, the receptor binding portion of human interleukin-2 can be combined with the envelope protein(s) of an alphavirus to target vectors to cells with IL-2 receptors. Furthermore, the foregoing technique can be used to create a recombinant alphavirus particles with envelope proteins that recognize Fc portions of antibodies. Monoclonal antibodies which recognize only preselected target cells are then bound to such Fc receptor-bearing alphavirus vector particles, such that the vector particles bind to and infect only those preselected target cells (for example, tumor cells). Alternatively, a hybrid envelope with the binding domain of avidin is used to target cells that have been coated 25

with biotinylated antibodies or other ligands. The patient is first flooded with antibodies, and then allowed time to clear unbound and nonspecifically-bound antibody before administering the vector. The high affinity ( $10^{-15}$ ) of the avidin binding site for biotin will allow accurate and efficient targeting to the original tissue identified by the monoclonal "image". Additional targeting approaches are known in the art and can readily be adopted for use in the practice of the present invention. For example, see U.S. Ser. No. 08/242, 407.

#### Example 10

##### LACTOSE FORMULATION OF A RECOMBINANT ALPHAVIRUS VECTOR

Crude recombinant alphavirus particles are obtained from a Celligan bioreactor (New Brunswick, N.J.) containing packaging cells transfected or transduced with the alphavirus vector construct, and bound to the beads of the bioreactor matrix. The cells release the packaged recombinant alphavirus particles into growth media that is passed over the cells in a continuous flow process. The media exiting the bioreactor is collected and passed initially through a 0.8 micron filter, then through a 0.65 micron filter to clarify the crude recombinant alphavirus particles. The filtrate is concentrated utilizing a cross flow concentrating system (Filtron, Boston, Mass.). Approximately 50 units of DNase (Intergen, New York, N.Y.) per ml of concentrate is added to digest exogenous DNA. The digest is diafiltrated using the same cross flow system to 150 mM NaCl, 25 mM tromethamine, pH 7.2. The diafiltrate is loaded onto a Sephadex S-500 gel column (Pharmacia, Piscataway, N.J.), equilibrated in 50 mM NaCl, 25 mM tromethamine, pH 7.4. The purified recombinant alphavirus particles are eluted from the Sephadex S-500 gel column in 50 mM NaCl, 25 mM tromethamine, pH 7.4. 30

The formulation buffer containing lactose is prepared as a 2x concentrated stock solution. The formulation buffer contains 25 mM tromethamine, 70 mM NaCl, 2 mg/ml arginine, 10 mg/ml human serum albumin (HSA), and 100 mg/ml lactose in a final volume of 100 mls at a pH 7.4. 35

The purified recombinant alphavirus particles are formulated by adding one part 2x lactose formulation buffer to one part S-500 purified recombinant alphavirus particle preparation. The formulated recombinant alphavirus particles can be stored at  $-70^{\circ}$  C. to  $-80^{\circ}$  C. or dried. 40

The formulated alphavirus particles are lyophilized in an Edwards Refrigerated Chamber (3 Shelf RC3S unit) attached to a Supermodulyo 12K freeze dryer (Edwards High Vacuum, Tonawanda, N.Y.). When the freeze drying cycle is completed, the vials are stoppered under a vacuum following a slight nitrogen gas bleeding. Upon removal, vials are crimped with aluminum seals. The lyophilized recombinant alphavirus particles are reconstituted with 1.0 ml water or other physiologically acceptable diluent. 45

#### Example 11

##### ADMINISTRATION OF RECOMBINANT ALPHAVIRUS PARTICLES

A therapeutic alphavirus vector used for the treatment of Gaucher disease (see Example 17) may be administered by transducing autologous CD34<sup>+</sup> cells in an ex vivo protocol or by direct injection of the vector into the patient's bone marrow. In order to achieve the longest therapeutic expression of GC from the recombinant multivalent vector, the best 50



mode of administration is to transduce long lived cell precursors of the clinically affected cell type, for example monocytes or macrophages. By transducing the earliest precursors of the effected cell type, the cell precursors are able to self renew and repopulate the peripheral blood with maturing GC positive cells. The earliest pluripotent hematopoietic stem cell studied to date are the CD34<sup>+</sup> cells which make up 1%–4% of a healthy bone marrow population or 0.1% in the peripheral blood population. Being able to transduce CD34<sup>+</sup> cells is important in sustaining long term expression not only for the monocyte/macrophage lineage but any hematopoietic cell targeted for a therapeutic protein. Two approaches for transducing CD34<sup>+</sup> cells include an ex vivo and an in vivo protocol. The in vivo protocol focuses on transducing an indiscriminate population of bone marrow cells by direct injection of the vector into the bone marrow of patients. The ex vivo protocol focuses on isolating CD34<sup>+</sup> positive stem cells, from the patient's bone marrow, or an infant patient's umbilical cord blood, transducing the cells with vector, then subsequently injecting the autologous cells back into the patient. Both approaches are feasible, but the ex vivo protocol enables the vector to be used most efficiently by transducing a specific cultured population of CD34<sup>+</sup> cells. Details of an ex vivo method are provided in the following section.

#### EX VIVO ADMINISTRATION OF A MULTIVALENT GC SINDBIS VECTOR

CD34<sup>+</sup> cells are collected from the patient's bone marrow by a syringe evacuation performed by a physician familiar with the technique. Alternatively, CD34<sup>+</sup> cells may also be obtained from an infant's umbilical cord blood if the patient is diagnosed before birth. Generally, if the bone marrow is the source of the CD34<sup>+</sup> cells, 20 bone marrow aspirations are obtained by puncturing femoral shafts or from the posterior iliac crest under local or general anesthesia. Bone marrow aspirations are then pooled, suspended in HEPES-Buffered Hanks' balanced salt solution containing heparin at 100 units per ml and deoxyribonuclease I at 100 ug/ml and then subjected to a Ficoll gradient separation. The buffy coated marrow cells are then collected and washed according to CellPro's CEPRATE® LC (CellPro, Bothell, Wash.) (CD34) Separation system (see U.S. Pat. Nos. 5,215,927; 5,225,353; 5,262,334; 5,215,926 and PCT/US91/07646). The washed buffy coated cells are then stained sequentially with anti-CD34 monoclonal antibody, washed then stained with biotinylated secondary antibody supplied with CEPRATE® system. The cell mixture is then loaded onto the CEPRATE® avidin column. The biotin-labeled cells are adsorbed onto the column while unlabeled cells passed through. The column is then rinsed according to the CEPRATE® system directions and CD34<sup>+</sup> cells eluted by agitation of the column by manually squeezing the gel bed. Once the CD34<sup>+</sup> cells are purified, the purified stem cells are counted and plated at a concentration of 1×10<sup>5</sup> cells/ml in Iscove's modified Dulbecco's medium (IMDM; Irvine Scientific, Santa Ana, Calif.) containing 20% pooled non-heat inactivated human AB serum (hAB serum).

After purification, several methods of transducing purified stem cells may be performed. One approach involves immediate transduction of the purified stem cell population with recombinant alphavirus particles contained in culture supernatants derived from vector packaging or producing cells. A second approach involves co-cultivation of an irradiated monolayer of vector producing cells with the purified population of nonadherent CD34<sup>+</sup> cells. A third approach involves a similar co-cultivation approach, however, the purified CD34<sup>+</sup> cells are prestimulated with various cytok-

ines and cultured 48 hours prior to the co-cultivation with the irradiated vector producing cells. Since alphavirus vectors are able to infect nonreplicating cells, prestimulation of these cells may not be required, however prestimulation of these cultures causing proliferation will provide increased cell populations for reinfusion into the patient.

Prestimulation of the CD34<sup>+</sup> cells is performed by incubating the cells with a combination of cytokines and growth factors which include IL-1, IL-3, IL-6 and mast cell growth factor (MGF). Prestimulation is performed by culturing 1–2×10<sup>5</sup> CD34<sup>+</sup> cells/ml of medium in T25 tissue culture flasks containing bone marrow stimulation medium for 48 hours. The bone marrow stimulation medium consists of IMDM containing 30% non-heat inactivated hAB serum, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 1M hydrocortisone, and 1% deionized bovine serum albumin. All reagents used in the bone marrow cultures should be screened for their ability to support maximal numbers of granulocyte, erythrocyte, macrophage, megakaryocyte, colony-forming units from normal marrow. Purified recombinant human cytokines and growth factors (Immune Corp., Seattle, Wash.) for prestimulation should be used at the following concentrations: *E. coli*-derived IL-1 (100 U/ml), yeast-derived IL-3 (5 ng/ml), IL-6 (50 U/ml), and MGF (50 ng/ml) (Anderson et al., *Cell Growth Differ.* 2:373, 1991).

After prestimulation of the CD34<sup>+</sup> cells, they are then infected by co-cultivation with the irradiated Sindbis producer cell line (expressing the GC therapeutic vector) in the continued presence of the stimulation medium. The Sindbis vector producing cell line is first trypsinized, irradiated (10,000 Rads) and replated at 1–2×10<sup>5</sup> cells/ml of bone marrow stimulation medium. The following day, 1–2×10<sup>5</sup> prestimulated CD34<sup>+</sup> cells/ml is added to the Sindbis vector producing cell line monolayer. Co-cultivation of the cells is performed for 48 hours. After co-cultivation, the CD34<sup>+</sup> cells are collected from the adherent Sindbis vector producing cell monolayer by vigorous washing with medium and plated for 2 hours to allow adherence of any dislodged vector producing cells. The CD34<sup>+</sup> cells are collected and expanded for an additional 72 hours. The cells are then harvested and frozen in liquid nitrogen using a cryoprotectant in aliquots of 1×10<sup>7</sup> cells per vial. Once the treated CD34<sup>+</sup> cells have been tested for the absence of adventitious agents, frozen transformed CD34<sup>+</sup> cells may be thawed, plated to a concentration of 1×10<sup>5</sup> cells/ml and cultured for an additional 48 hours in bone marrow stimulation medium. Transformed cells are collected, washed twice and resuspended in normal saline. The number of transduced cells used to infuse back into the patient per infusion is projected to be at a minimum of 1–10×10<sup>7</sup> cells per patient per injection site requiring up to four injection sites. Infusion may be performed directly back into the patient's bone marrow or directly into the peripheral blood stream. Patients receiving autologous transduced bone marrow cells may be either partially or whole body irradiated, to deplete existing bone marrow populations. Treatment may be assessed at various time points post infusion to determine GC activity and for length of expression in differentiated cell types. If at some point during the course of follow-up procedures expression decreases or is nonexistent, transduced autologous cells may be reinjected into the patient.



**DETERMINATION OF VECTOR UNITS IN A  
PREPARATION BY INFECTION OF A  
REPORTER PROTEIN EXPRESSING CELL  
LINE UNDER THE CONTROL OF THE  
SINDBIS JUNCTION REGION**

**DETERMINATION OF VECTOR UNITS IN A  
PREPARATION BY INFECTION OF A  $\beta$ -  
GALACTOSIDASE EXPRESSING REPORTER  
CELL LINE**

In order to administer the proper therapeutic dose of vector to individuals, it is desirable to derive a method by which the vector infectious units contained in a preparation can be determined easily. This is accomplished by the generation of a cell line which expresses  $\beta$ -galactosidase or another reporter gene only when functional Sindbis non-structural proteins are present in the cell. The cell line can be infected with increasing dilutions of a Sindbis vector preparation such that individual cells are not infected with more than one vector particle, allowing the titer, or vector units, to be determined. Thus, the cell line is an assay of functional particles present in a vector preparation.

**A. GENERATION OF A CELL LINE WHICH  
EXPRESSES FUNCTIONAL  $\beta$ -GALACTOSIDASE PRO-  
TEIN UNDER THE CONTROL OF SINDBIS NON-  
STRUCTURAL PROTEINS**

In one configuration, a eukaryotic expression cassette is constructed which contains a 5'-end sequence capable of initiating transcription of Sindbis RNA, a Sindbis junction region, a reporter gene, and a 3'-end Sindbis RNA polymerase recognition sequence for minus-strand synthesis. This cassette is positioned in an antisense orientation, adjacent to a eukaryotic transcriptional promoter. Additionally, these constructs also may contain a catalytic ribozyme sequence immediately adjacent to Sindbis nucleotide 1. of the 5'-end sequence which will result in cleavage of the primary RNA transcript precisely after this Sindbis nucleotide. In this antisense orientation, the reporter gene cannot be translated and is dependent entirely on the presence of Sindbis nonstructural proteins for transcription into positive stranded mRNA prior to reporter gene expression. These non-structural proteins will be provided by the Sindbis vector preparation being titered. In addition, this configuration, if designed to contain the precise Sindbis genome 5'- and 3'-end sequences, will allow for the reporter gene transcripts to undergo amplification by utilizing the same nonstructural proteins provided by the Sindbis vector.

An example of this antisense titration construction is as follows. Briefly, the plasmid pKSSINBV-lacZ (described in Example 6) is digested with the enzymes Apa I and Bam HI. This results in the removal of the Sindbis 5' and Sindbis nonstructural protein sequences. The 7 kbp fragment is purified on a 0.7% agarose gel. This fragment is ligated to a fragment obtained by digestion of pdS26s (described in Example 7) with Apa I and BamHI followed by gel purification of the 0.4kbp fragment containing the HDV ribozyme and 5' Sindbis sequences. The resulting construct is known as pKSd5'BV-lacZ. pKSd5'BV-lacZ is digested with Apa I and Pme I followed by purification of the 7.4kbp fragment on a 0.7% agarose gel. This fragment contains the HDV ribozyme, Sindbis 5' end, junction region, LacZ gene, and Sindbis 3' end sequences. This fragment is ligated in the antisense orientation into pcDNA3 (Promega Corp., Madison, Wis.) by digestion of pcDNA3 with Apa I and EcoRV followed by GENECLAN™ purification. The

resulting construct, containing a CMV promoter which transcribes an antisense reporter cassette RNA of the configuration Sindbis 3'-end sequence/LacZ gene/junction region/Sindbis 5'-end sequence/HDV ribozyme, is known as pSINjra-gal.

BHKSINjra-gal cells are derived by transfection of  $5 \times 10^5$  BHK-21 cells, grown in a 60 mm petri dish, with 5  $\mu$ g of the pSINjra-gal vector complexed with the polycation reagent Transfectam™ (Promega, Madison, Wis.). At 24 hour post-transfection, the media is supplemented with 400  $\mu$ g/ml of G418 (GibcoBRL, Gaithersburg, Md.). After all non-transfected cells have died and G418 resistant colonies have begun dividing, the cells are removed from the plate by trypsinization, pooled, then cloned by limiting dilution. Several clones are tested for the production of functional  $\beta$ -galactosidase by infection with a known titer of a wild-type stock of Sindbis virus. Production of functional  $\beta$ -galactosidase in candidate BHKSINjra-gal clones is determined 6 hours post-infection by first fixing PBS-rinsed cells with a solution containing 2% formaldehyde (37% stock solution)/0.2% glutaraldehyde, then staining the cells with a solution containing 0.5 mM potassium ferricyanide/0.5 mM potassium ferrocyanide/2 mM  $MgCl_2$ /1 mg/ml X-gal. Blue cells are clearly visible within 3 hours. Provided that the Sindbis virus stock does not contain a high level of defective interfering (DI) particles, the virus titer as determined by plaque assay on BHK-21 cells should be similar to the titer observed by X-gal staining on BHKSINjra-gal cells.

The titer of various alphavirus vector preparations, in vector units, produced from packaging cell lines such as those described in Example 7, is determined by infection of confluent monolayers of BHKSINjra-gal cells with several dilutions of vector. The titer of the vector preparation is determined at 6 hour post-infection by visualization of cells producing  $\beta$ -galactosidase protein, as described above. Since the alphavirus vectors described do not contain the viral region corresponding to the structural genes, it is not possible to determine the titer of a vector preparation by plaque assay in BHK-21 cells.

Alternatively, a titrating cell line is produced by using a different reporter cassette configuration, which consists of a eukaryotic promoter/5'-end Sindbis sequence recognized by the viral transcriptase/Sindbis junction region/reporter gene/Sindbis RNA polymerase recognition sequence for minus-strand synthesis, and is expressed in a sense-orientation. This reporter expression cassette requires synthesis, by vector-supplied Sindbis nonstructural proteins, into an antisense RNA molecule, prior to transcription of the subgenomic message encoding the reporter gene.

Specifically, the sense-orientation packaging construct is created as follows. Plasmid pVGELVIS is digested with the enzyme Apa I, which cleaves at nucleotide 11737, just downstream of the Sindbis 3'-end. The Apa I-digested DNA is blunt-ended by the addition of T4 DNA polymerase and dNTPs and incubation at 16° C. for 10 minutes. After heat inactivation of the polymerase, the DNA fragment is digested with the enzyme Sfi I, and the 10041 bp fragment is purified in a 1% agarose gel. Plasmid pKSSINBV-lacZ is digested with the enzymes Pme I and Sfi I. The 6.4 kbp fragment is purified in a 1% agarose gel. The 6.4 kbp pKSSINBV-lacZ fragment then is ligated into the purified pVGELVIS fragment to create the plasmid pELVIS-gal. This plasmid contains the complete Sindbis nonstructural proteins, Sindbis junction region, LacZ gene and Sindbis 3'-end replicase recognition sequence under the control of the MuLV LTR promoter. Plasmid pELVIS-gal is digested with Bsp EI, purified by GENECLAN (Bio 101 Corp., San Diego, Calif.) and religated to itself. Bsp EI removes the Sindbis nonstructural protein gene sequences between nts

422-7054. The re-ligated construct contains a 5' sequence that is capable of initiating transcription of Sindbis RNA, Sindbis junction region, sequences encoding the LacZ gene, and Sindbis 3'-end sequences required for synthesis of the minus-strand RNA, all downstream, and under the transcriptional control of a MuLV-LTR promoter. This construct is known as pELVISdINSP-gal.

Plasmid pELVISdINSP-gal is transfected into BHK-21 cells and tested as described previously. The BHK pELVISdINSP-gal cells produces an RNA transcript with a 5'-end sequence that is recognized by the Sindbis transcriptase, a Sindbis junction region, sequences encoding the LacZ gene, and Sindbis 3'-end sequences required for synthesis of the minus-strand RNA. 8-galactosidase expression from the primary transcript is prevented because of an upstream open-reading frame and stop codons created by the Bsp EI deletion. The addition of Sindbis nonstructural proteins, provided by the Sindbis vector being titrated, results in transcription of active LacZ transcripts from the Sindbis junction region, after initial synthesis of an antisense intermediate. Furthermore, this configuration, if designed to contain the precise Sindbis genome 5'- and 3'-end sequences, allows the reporter gene transcripts to undergo amplification by utilizing the same nonstructural proteins provided by the Sindbis vector.

In another configuration, a titrating cell line is produced using an expression cassette containing an antisense reporter gene followed by the 3'-end alphavirus replicase recognition sequences, positioned in the sense-orientation. This construct, under the control of a eukaryotic promoter, produces an RNA transcript that is recognized and transcribed by alphavirus nonstructural proteins provided by the vector to be titrated. The alphavirus nonstructural proteins recognize sequences in the primary reporter transcript, and in turn, synthesize a sense reporter transcript. This construct does not benefit from amplification of the reporter gene transcript, but should still provide sufficient transcripts to allow for vector titrating.

Construction of this type of titrating cassette is as follows. Briefly, pSV- $\beta$ -galactosidase vector (Promega Corp., Madison, Wis.) is digested with the enzyme Hind III and blunt-ended as described above. The plasmid is further digested with the enzymes Bam HI and Xmn I to remove the LacZ gene, and reduce the size of the remaining fragment. The 3737 nt fragment, containing the LacZ gene, is purified in a 1% agarose gel and ligated into pcDNA3 (Invitrogen, San Diego, Calif.) that has been digested with the enzymes Bam HI and Eco RV. The new plasmid construct is known as pcDNAaLacZ. This plasmid is digested with the enzyme Apa I, blunt-ended as above, and further digested with the enzyme Xho I. Plasmid pSKSINBV (described previously) is digested with Sac I, blunt-ended as before, and then digested with Xho I. The resulting 146 nt fragment containing the Sindbis 3' replicase recognition sequence is purified in a 1.2% agarose gel, ligated into the digested pcDNAaLacZ vector. The re-ligated construct contains an antisense LacZ gene and a 3' Sindbis replicase protein recognition sequence downstream from a CMV promoter. The resulting construct is known as pcDNAaLacZ-3'Sin. The construct is transfected into BHK cells and utilized as described previously.

#### B. GENERATION OF A CELL LINE WHICH EXPRESSES FUNCTIONAL LUCIFERASE PROTEIN UNDER THE CONTROL OF SINDBIS NONSTRUCTURAL PROTEINS.

An alternate reporter for a titrating construct based upon the sense configuration of the reporter gene and requiring the nonstructural proteins for expression utility is luciferase. Again, the non-structural proteins are supplied in trans by the Sindbis vector preparation being titrated. To generate this construct, pELVIS-luc is digested with Eco 47 III and Hpa

I. These digests remove nucleotides 1407-6920 from within the non-structural coding region. After heat inactivation of the enzymes, the digested vector is religated under dilute conditions. This construct is known as pELVISdIE-Hluc. The construct is transfected into BHK cells and utilized as described previously.

#### Example 13

#### GENERATION OF VECTOR CONSTRUCTS WHICH EXPRESS HBV ANTIGENS FOR THE INDUCTION OF AN IMMUNE RESPONSE

##### A. ISOLATION OF HBV E/CORE SEQUENCE

A 1.8 Kb fragment containing the entire precore/core coding region of hepatitis B is obtained from plasmid pAM6 (ATCC No 45020) following Bam HI digestion and gel purification, and ligated into the Bam HI site of KS II+ (Stratagene, La Jolla, Calif.). This plasmid is designated KS II+ HBpc/c. Xho I linkers are added to the Stu I site of precore/core in KS II+ HBpc/c (at nucleotide sequence 1,704), followed by cleavage with Hinc II (at nucleotide sequence 2,592). The resulting 877 base pair Xho I-Hinc II precore/core fragment is cloned into the Xho I/Hinc II site of SK II+. This plasmid is designated SK+HBc.

##### B. PREPARATION OF SEQUENCES UTILIZING PCR

##### 1. SITE-DIRECTED MUTAGENESIS OF HBV E/CORE SEQUENCE UTILIZING PCR

The precore/core gene in plasmid KS II+ HB pc/c is sequenced to determine if the precore/core coding region is correct. This sequence was found to have a single base-pair deletion which causes a frame shift at codon 79 that results in two consecutive in-frame TAG stop codons at codons 84 and 85. This deletion is corrected by PCR overlap extension (Ho et al., *Gene* 77:51, 1989) of the precore/core coding region in plasmid SK+ HBc. Four oligonucleotide primers are used for the 3 PCR reactions performed to correct the deletion.

The first reaction utilizes two primers. The sense primer sequence corresponds to the nucleotide sequence 1,805 to 1,827 of the adw strain and contains two Xho I restriction sites at the 5' end. The nucleotide sequence numbering is obtained from Genbank (Intelligenics, Inc., Mountain View, Calif.).

5' CTC GAG CTC GAG GCA CCA GCA CCA TGC AAC TTT TT-3' (SEQ. ID NO.92)

The second primer sequence corresponds to the anti-sense nucleotide sequence 2,158 to 2,130 of the adw strain of hepatitis B virus, and includes codons 79, 84 and 85.

5'-CTA CTA GAT CCC TAG ATG CTG GAT CTT CC-3' (SEQ. ID NO.93)

The second reaction also utilizes two primers. The sense primer corresponds to nucleotide sequence 2,130 to 2,158 of the adw strain, and includes codons 79, 84 and 85.

5'-GGA AGA TCC AGC ATC TAG GGA TCT AGT AG-3' (SEQ. ID NO.94)

The second primer corresponds to the anti-sense nucleotide sequence from SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop codon of the HBV precore/core coding region.

5'-GGG CGA TAT CAA GCT TAT CGA TAC CG-3' (SEQ. ID NO.95)

The third reaction also utilizes two primers. The sense primer corresponds to nucleotide sequence 5 to 27 of the adw strain, and contains two Xho I restriction sites at the 5' end.

5'- CTC GAG CTC GAG GCA CCA GCA CCA TGC AAC TTT TT (SEQ. ID NO.92)

The second primer sequence corresponds to the anti-sense nucleotide sequence from the SK+ plasmid polylinker and contains a Cla I site 135 bp downstream of the stop codon of the HBV precore/core coding region.

5'-GGG CGA TAT CAA GCT TAT CGA TAC CG-3' (SEQ. ID NO.96)

The first PCR reaction corrects the deletion in the anti-sense strand and the second reaction corrects the deletion in the sense strands. PCR reactions one and two correct the mutation from CC to CCA which occurs in codon 79 and a base pair substitution from TCA to TCT in codon 81. Primer 1 contains two consecutive Xho I sites 10 bp upstream of the ATG codon of HBV e coding region and primer 4 contains a Cla I site 135 bp downstream of the stop codon of HBV precore/core coding region. The products of the first and second PCR reactions are extended in a third PCR reaction to generate one complete HBV precore/core coding region with the correct sequence.

The PCR reactions are performed using the following cycling conditions: The sample is initially heated to 94° C. for 2 minutes. This step, called the melting step, separates the double-stranded DNA into single strands for synthesis. The sample is then heated at 56° C. for 30 seconds. This step, called the annealing step, permits the primers to anneal to the single stranded DNA produced in the first step. The sample is then heated at 72° C. for 30 seconds. This step, called the extension step, synthesizes the complementary strand of the single stranded DNA produced in the first step. A second melting step is performed at 94° C. for 30 seconds, followed by an annealing step at 56° C. For 30 seconds which is followed by an extension step at 72° C. for 30 seconds. This procedure is then repeated for 35 cycles resulting in the amplification of the desired DNA product.

The PCR reaction product is purified by 1.5% agarose gel electrophoresis and transferred onto NA 45 paper (Schleicher and Schuell, Keene, N.H.). The desired 787 bp DNA fragment is eluted from the NA 45 paper by incubating for 30 minutes at 65° C. in 400 l high salt buffer (1.5M NaCl, 20 mM Tris, pH 8.0, and 0.1 mM EDTA). Following elution, 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1) is added to the solution. The mixture is vortexed and then centrifuged 14,000 rpm for 5 minutes in a Brinkmann Eppendorf centrifuge (5415L). The aqueous phase, containing the desired DNA fragment, is transferred to a fresh 1.5 ml microfuge tube and 1.0 ml of 100% EtOH is added. This solution is incubated on dry ice for 5 minutes, and then centrifuged for 20 minutes at 10,000 rpm. The supernatant is decanted, and the pellet is rinsed with 500 µl of 70% EtOH. The pellet is dried by centrifugation at 10,000 rpm under vacuum, in a Savant Speed-Vac concentrator, and then resuspended in 10 l deionized H<sub>2</sub>O. One microliter of the PCR product is analyzed by 1.5% agarose gel electrophoresis. The 787 Xho I-Cla I precore/core PCR amplified fragment is cloned into the Xho I-Cla I site of SK+ plasmid. This plasmid is designated SK+HBc-c. *E. coli* (DH5 alpha, Bethesda Research Labs, Gaithersburg, Md.) is transformed with the SK+HBc-c plasmid and propagated to generate plasmid DNA. The plasmid is then isolated and purified, essentially as described by Birnboim et al. (*Nuc. Acid Res.* 7:1513, 1979; see also *Molecular Cloning: A Laboratory Manual*, Sambrook et al. (eds.), Cold Spring Harbor Press, 1989). The SK+HBc-c plasmid is analyzed to confirm the sequence of the precore/core gene (FIG. 4).

## 2. ISOLATION OF HBV CORE SEQUENCE

The single base pair deletion in plasmid SK+ HBc is corrected by PCR overlap extension as described above in Example 13B. Briefly, four oligonucleotide primers are used for the PCR reactions performed to correct the mutation.

The first reaction utilizes two primers. The sense primer corresponds to the nucleotide sequence for the T-7 promoter of SK+HBc plasmid.

5'-AAT ACG ACT CAC TAT AGG G-3' (SEQ. ID NO. 97)

The second primer corresponds to the anti-sense sequence 2,158 to 2,130 of the adw strain, and includes codons 79, 84 and 85.

5'-CTA CTA GAT CCC TAG ATG CTG GAT CTT CC-3' (SEQ. ID NO. 98)

The second reaction utilizes two primers. The anti-sense primer corresponds to the nucleotide sequence for the T-3 promoter present in SK+HBc plasmid.

5'-3': ATT AAC CCT CAC TAA AG (SEQ. ID NO. 99)

The second primer corresponds to the sense nucleotide sequence 2,130 to 2,158 of the adw strain, and includes codons 79, 84 and 85.

5'-GGA AGA TCC AGC ATC TAG GGA TCT AGT AG-3' (SEQ. ID NO. 100)

The third reaction utilizes two primers. The anti-sense primer corresponds to the nucleotide sequence for the T-3 promoter present in SK+HBc plasmid.

5'-ATT AAC CCT CAC TAA AG-3' (SEQ. ID NO. 101)

The second primer corresponds to the sense sequence of the T-7 promoter present in the SK+HBc plasmid.

5'-AAT ACG ACT CAC TAT AGGOG-3' (SEQ. ID NO.102)

The PCR product from the third reaction yields the correct sequence for HBV precore/core coding region.

To isolate HBV core coding region, a primer is designed to introduce the Xho I restriction site upstream of the ATG start codon of the core coding region, and eliminate the 29 amino acid leader sequence of the HBV precore coding region. In a fourth reaction, the HBV core coding region is produced using the PCR product from the third reaction and the following two primers.

The sense primer corresponds to the nucleotide sequence 1,885 to 1,905 of the adw strain and contains two Xho I sites at the 5' end.

5'-CCT CGA GCT CGA GCT TGG GTG GCT TTG GGG CAT G-3' (SEQ. ID NO.103)

The second primer corresponds to the anti-sense nucleotide sequence for the T-3 promoter present in the SK+HBc plasmid. The approximately 600 bp PCR product from the fourth PCR reaction contains the HBV core coding region and novel Xho I restriction sites at the 5' end and Cla I restriction sites at the 31 end that was present in the multicloning site of SK+HBc plasmid.

5'-ATF ACC CCT CAC TAA AG-3' (SEQ. ID NO.104)

Following the fourth PCR reaction, the solution is transferred into a fresh 1.5 ml microfuge tube. Fifty microliters of 3M sodium acetate is added to this solution followed by 500 µl of chloroform: isoamyl alcohol (24:1). The mixture is vortexed and then centrifuged at 14,000 rpm for 5 minutes. The aqueous phase is transferred to a fresh microfuge tube and 1.0 ml 100% EtOH is added. This solution is incubated at -20° C. for 4.5 hours, and then centrifuged at 10,000 rpm for 20 minutes. The supernatant is decanted, and the pellet is rinsed with 500 µl of 70% EtOH. The pellet is dried by centrifugation at 10,000 rpm under vacuum and then resuspended in 10 µl deionized H<sub>2</sub>O. One microliter of the PCR product is analyzed by 1.5% agarose gel electrophoresis. The approximately 600 bp Xho I-Cla I HBV core PCR fragment is cloned into the Xho I-Cla I site of SK+ plasmid. This plasmid is designated SK+HBc.

### 3. ISOLATION OF HBV X ANTIGEN

A 642 bp Nco I - Taq I fragment containing the hepatitis B virus X open reading frame is obtained from the pAM6 plasmid (adw) (ATCC 45020), blunted by Klenow fragment, and ligated into the Hinc II site of SK<sup>+</sup> (Stratagene, La Jolla, Calif.). *E. coli* (DH5<sup>+</sup>, Bethesda Research Laboratories, Gaithersburg, Md.) is transformed with the ligation reaction and propagated.

Since this fragment can be inserted in either orientation, clones are selected that have the sense orientation with respect to the Xho I and Cla I sites in the SK<sup>+</sup> multicloning site. More specifically, miniprep DNAs are digested with the diagnostic restriction enzyme, Bam HI. Inserts in the correct orientation yield two fragments of 3.0 Kb and 0.6 Kb in size. Inserts in the incorrect orientation yield two fragments of 3.6 Kb and 0.74 Kb. A clone in the correct orientation is selected and designated SK-X Ag.

### 4. CONSTRUCTION OF SINDBIS VECTORS EXPRESSING HBVE, HBV CORE AND HBV X

Construction of a Sindbis vector expressing the HBV e sequence is accomplished by digesting the SK<sup>+</sup>HB e-c plasmid with Xho I and Xba I to release the cDNA fragment encoding HBV e-c sequences. The fragment is then isolated by agarose gel electrophoresis, purified by GENECLEAN<sup>™</sup>, and inserted into pKSSINBV (see Example 3), prepared by digestion with Xho I and Xba I, and treated with CIAP. This vector is designated pKSSIN-HBc. Similar vectors may also be made from other Sindbis vectors described in Example 3, such as, for example, pKSSINd1JRsirc, pKSSINd1JRsirPC, pKSSINd1JRsirNP (7582-7601) and pKSSINd1JRsirx.

Construction of a Sindbis vector expressing the HBV core sequence is accomplished by digestion of plasmid SK<sup>+</sup>HBc (described above) with Xho I and Xba I. The HBc fragment is isolated by agarose gel electrophoresis, purified by GENECLEAN<sup>™</sup> and ligated into pKSSINBV at the Xho I and Xba I sites. This Sindbis-HBc vector is designated pKSSIN-HBc.

Construction of a Sindbis vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Xba I to release a cDNA fragment encoding HBV-X sequences. The fragment is isolated by agarose gel electrophoresis, purified using GENECLEAN<sup>™</sup>, and inserted into pKSSINBV, pre-treated with Xho I and Xba I enzymes. This Sindbis-HBx vector is designated pKSSIN-HBx.

The above Sindbis HBV expressing vectors may also be modified to coexpress a selectable drug resistance marker dependent on the requirements of the experiment or treatment of the vector infected cells. In particular, any of the above Sindbis HBV expression vectors described may also be designed to coexpress G418 resistance. This is accomplished by incorporating an internal ribosomal entry site (Example 5) followed by the bacterial neomycin phosphotransferase gene placed 3' of the HBV coding sequences and 5' of the terminal 3' end of the vector using the multiple cloning site of the vector. These G418 resistant vector constructs can be used for selecting vector infected cells for the generation of HBV specific CTL targets in the following sections.

### D. EXPRESSION IN INFECTED CELLS WITH SINDBIS VECTORS

#### 1. ELISA

Cell lysates from cells infected by any of the HBV expressing vectors are made by washing  $1.0 \times 10^7$  cultured cells with PBS, resuspending the cells to a total volume of 600  $\mu$ l in PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350 (Fisher,

Pittsburgh, Pa.) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

Core antigen and precore antigen in cell lysates and secreted e antigen in culture supernatant are assayed using the Abbott HBe, rDNA E1A kit (Abbott Laboratories Diagnostic Division, Chicago, Ill.). Another sensitive EIA assay for precore antigen in cell lysates and secreted e antigen in culture supernatant is performed using the Incstar ETI-EB kit (Incstar Corporation, Stillwater, Minn.). A standard curve is generated from dilutions of recombinant hepatitis B core and e antigen obtained from Biogen (Geneva, Switzerland).

As shown in FIG. 16, using these procedures approximately 100-200 ng/ml HBV e antigen is expressed in the cell lysates and 300-400 ng/ml HBV e antigen is secreted from BHK cells infected with the Sin BV HB e vector.

As shown in FIG. 17, using these procedures, approximately 40 ng/ml HBV core antigen is expressed in the cell lysates from  $10^6$  BHK cells infected with the Sin BV HBcore. Mouse fibroblast cells infected with the recombinant HBcore Sindbis vector express 6-7 fold higher HBV core protein levels than the recombinant HBcore retroviral vector transduced cells (WO 93/15207). As shown in FIG. 18, using these procedures, approximately 12-14 ng/ml HBV core antigen is expressed in the cell lysates from  $10^6$  L-M(TK<sup>-</sup>) cells infected with the SinBVHBcore vector as compared to the approximately 2 ng/ml HBV core antigen expressed from recombinant HBcore retroviral vector transducer cells.

### 2. IMMUNOPRECIPITATION/WESTERN BLOT

Characterization of the precore/core and e antigens expressed by vector infected cells is performed by immunoprecipitation followed by Western blot analysis. Specifically, 0.5-1.0 ml of cell lysate in PBS or culture supernatant is mixed with polyclonal rabbit anti-hepatitis B core antigen (DAKO Corporation, Carpinteria, Calif.) bound to protein G-Sepharose (Pharmacia LKB, Uppsala, Sweden) and incubated overnight at 4° C. Samples are washed twice in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA and boiled in sample loading buffer with 0.5% 2-mercaptoethanol. Proteins are first resolved by SDS polyacrylamide gel electrophoresis, and then transferred to Immobilon (Millipore Corp., Bedford, Me.) and probed with the DAKO polyclonal rabbit anti-hepatitis B core antigen, followed by <sup>125</sup>I-protein A.

### E. TESTING IMMUNE RESPONSE

#### 1. CYTOTOXICITY ASSAYS

##### (a) Inbred Mice

Six- to eight-week-old female C3H/He mice (Charles River, Mass.) are injected twice intraperitoneally (i.p.) at 1 week intervals with  $1 \times 10^6$  of Sindbis HBe or HBcore vector. Animals are sacrificed 7 or 14 days later and the splenocytes ( $3 \times 10^6$ /ml) cultured in vitro with their respective irradiated ( $10,000$  rads) retroviral vector transduced cells ( $6 \times 10^4$ /ml) (WO 93/15207) in T-25 flasks (Corning, Corning, N.Y.). Culture medium consists of RPMI 1640, 5% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 50  $\mu$ g/ml gentamycin and  $10^{-5}$ M 2-mercaptoethanol (Sigma, St. Louis, Mo.). Effector cells are harvested 4-7 days later and tested using various effector:target cell ratios in 96 well microtiter plates (Corning, Corning, N.Y.) in a standard chromium release assay. Targets are the retroviral vector the retroviral vector transduced L-M(TK<sup>-</sup>) cells (ATCC No. CCL 1.3) whereas the non-transduced syngeneic cell lines are used as negative controls. CTL targets may also be generated by infecting syngeneic cells with the Sindbis HBe or HBcore vector coexpressing the G418 resistance marker. Infected cells are then selected using 800 g/ml G418 for two

weeks. Specifically,  $\text{Na}_2^{51}\text{CrO}_4$ -labeled (Amersham, Arlington Heights, Ill.) (100  $\mu\text{Ci}$ , 1 hour at  $37^\circ\text{C}$ ) target cells ( $1 \times 10^4$  cells/well) are mixed with effector cells at various effector to target cell ratios in a final volume of 200  $\mu\text{l}$ . Following incubation, 100  $\mu\text{l}$  of culture medium is removed and analyzed in a Beckman gamma spectrometer (Beckman, Dallas, Tex.). Spontaneous release (SR) is determined as CPM from targets plus medium and maximum release (MR) is determined as CPM from targets plus 1M HCl. Percent target cell lysis is calculated as:  $[(\text{Effector cell} + \text{target CPM}) - (\text{SR}) / (\text{MR}) - (\text{SR})] \times 100$ . Spontaneous release values of targets are typically 10%–20% of the MR.

For certain CTL assays, the effectors may be in vitro stimulated multiple times, for example, on day 8–12 after the primary in vitro stimulation. More specifically,  $10^7$  effector cells are mixed with  $6 \times 10^5$  irradiated (10,000 rads) stimulator cells, and  $2 \times 10^7$  irradiated (3,000 rads) "filler" cells (prepared as described below) in 10 ml of "complete" RPMI medium. (RPMI containing: 5% heat inactivated Fetal Bovine Serum, two mM L-glutamine, 1 mM sodium pyruvate, 1X non essential amino acids, and  $5 \times 10^{-5}\text{M}$  2-mercaptoethanol). Stimulator cells for in vitro stimulation of effector cells are generated from irradiated retroviral vector transduced (10,000 rads) L-M (TK<sup>-</sup>) cells. "Filler" cells are prepared from naive syngeneic mouse spleen cells resuspended in RPMI, irradiated with 3,000 rads at room temperature. Splenocytes are washed with RPMI, centrifuged at 3,000 rpm for 5 minutes at room temperature, and the pellet is resuspended in RPMI. The resuspended cells are treated with 1.0 ml tris-ammonium chloride (100 ml of 0.17 M tris base, pH 7.65, plus 900 ml of 0.155 M  $\text{NH}_4\text{Cl}$ ; final solution is adjusted to a pH of 7.2) at  $37^\circ\text{C}$  for 3–5 minutes. The secondary in vitro restimulation is then cultured for 5–7 days before testing in a CTL assay. Any subsequent restimulations are cultured as described above with the addition of 2–10 U of recombinant human IL-2 (200 U/ml, catalog #799068, Boehringer Mannheim, W. Germany).

Using these procedures, it can be shown that CTLs to HBV e antigen can be induced.

#### (b) HLA A2.1 Transgenic Mice

Six- to eight-week-old female HLA A2.1 transgenic mice (V. Engelhard, Charlottesville, Va.) are injected twice intraperitoneally (i.p.) at one week intervals with  $1.0 \times 10^6$  pfu of Sindbis vector expressing HBe or HBcore. Animals are sacrificed 7 days later and the splenocytes ( $3 \times 10^6/\text{ml}$ ) cultured in vitro with irradiated (10,000 rads) retroviral vector transduced Jurkat A2/K<sup>b</sup> cells (WO 93/15207), or with peptide coated Jurkat A2/K<sup>b</sup> cells ( $6 \times 10^4/\text{ml}$ ) in flasks (T-25, Corning, Corning, N.Y.). The remainder of the chromium release assay is performed as described in Example 13E 1.a, where the targets are transduced and non-transduced EL4 A2/K<sup>b</sup> (WO 93/15207) and Jurkat A2/K<sup>b</sup> cells. Non-transduced cell lines are utilized as negative controls. The targets may also be peptide coated EL4 A2/K<sup>b</sup> cells.

#### (c) Transduction of Human Cells With Vector Construct

Lymphoblastoid cell lines (LCL) are established for each patient by infecting (transforming) their B-cells with fresh Epstein-Barr virus (EBV) taken from the supernatant of a 3-week-old culture of B95-8, EBV transformed marmoset leukocytes (ATCC CRL 1612). Three weeks after EBV transformation, the LCL are infected with Sindbis vector expressing HBV core or e antigen and G418 resistance. Vector infection of LCL is accomplished by infecting LCL cells with packaged alphavirus vector particles produced from the appropriate cell line. The culture medium consists of RPMI 1640, 20% heat inactivated fetal bovine serum (Hyclone, Logan, UT), 5.0 mM sodium pyruvate and 5.0

mM non-essential amino acids. Infected LCL cells are selected by adding 800  $\mu\text{g}/\text{ml}$  G418. The Jurkat A2/K<sup>b</sup> cells (L. Sherman, Scripps Institute, San Diego, Calif.) are infected essentially as described for the infection of LCL cells.

#### (d) Human CTL assays

Human PBMC are separated by Ficoll (Sigma, St. Louis, Mo.) gradient centrifugation. Specifically, cells are centrifuged at 3,000 rpm at room temperature for 5 minutes. The PBMCs are restimulated in vitro with their autologous retroviral vector transduced (WO 93/15207) LCL or HLA-matched cells at an effector:target ratio of 10:1 for 10 days. Culture medium consists of RPMI 1640 with prescreened lots of 5% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate and 50  $\mu\text{g}/\text{ml}$  gentamycin. The resulting stimulated CTL effectors are tested for CTL activity using Sindbis vector infected autologous LCL or HLA-matched cells as targets in the standard chromium release assay, Example 13 1.a. Since most patients have immunity to EBV, the non-transduced EBV-transformed B-cells (LCL) used as negative controls, will also be recognized as targets by EBV-specific CTL along with the transduced LCL. In order to reduce the high background due to killing of labeled target cells by EBV-specific CTL, it is necessary to add unlabeled non-transduced LCL to labeled target cells at a ratio of 50:1.

#### 2. DETECTION OF HUMORAL IMMUNE RESPONSE

Humoral immune responses in mice specific for HBV core and e antigens are detected by ELISA. The ELISA protocol utilizes 100  $\mu\text{g}/\text{well}$  of recombinant HBV core and recombinant HBV e antigen (Biogen, Geneva, Switzerland) to coat 96-well plates. Sera from mice immunized with vector expressing HBV core or HBV e antigen are then serially diluted in the antigen-coated wells and incubated for 1 to 2 hours at room temperature. After incubation, a mixture of rabbit anti-mouse IgG1, IgG2a, IgG2b, and IgG3 with equivalent titers is added to the wells. Horseradish peroxidase ("HRP")-conjugated goat anti-rabbit anti-serum is added to each well and the samples are incubated for 1 to 2 hours at room temperature. After incubation, reactivity is visualized by adding the appropriate substrate. Color will develop in wells that contain IgG antibodies specific for HBV core or HBV e antigen.

#### 3. T CELL PROLIFERATION

Antigen induced T-helper activity resulting from two or three injections of Sindbis vector expressing HBV core or e antigen, is measured in vitro. Specifically, splenocytes from immunized mice are restimulated in vitro at a predetermined ratio with cells expressing HBV core or e antigen or with cells not expressing HBV core or e antigen as a negative control. After five days at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  in RPMI 1640 culture medium containing 5% FBS, 1.0 mM sodium pyruvate and  $10^{-5}$  2-mercaptoethanol, the supernatant is tested for IL-2 activity. IL-2 is secreted specifically by T-helper cells stimulated by HBV core or e antigen, and its activity is measured using the CTL clone, CTLL-2 (ATCC TIB 214). Briefly, the CTLL-2 clone is dependent on IL-2 for growth and will not proliferate in the absence of IL-2. CTLL-2 cells are added to serial dilutions of supernatant test samples in a 96-well plate and incubated at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$  for 3 days. Subsequently, 0.5  $\mu\text{Ci}$   $^3\text{H}$ -thymidine is added to the CTLL-2 cells. 0.5  $\mu\text{Ci}$   $^3\text{H}$ -thymidine is incorporated only if the CTLL-2 cells proliferate. After an overnight incubation, cells are harvested using a PHD cell harvester (Cambridge Technology Inc., Watertown, Mass.) and counted in a Beckman beta counter. The amount of IL-2 in a sample is determined from a standard curve generated from a recombinant IL-2 standard obtained from Boehringer Mannheim (Indianapolis, Ind.).

## F. ADMINISTRATION PROTOCOLS

## 1. MICE

## (a) Direct Vector Administration

The mouse system may also be used to evaluate the induction of humoral and cell-mediated immune responses with direct administration of Sindbis vector encoding HBV core or e antigen. Briefly, six- to eight-week-old female C3H/He mice are injected intramuscularly (i.m.) with 0.1 ml of reconstituted (with sterile deionized, distilled water) or intraperitoneally (ip) with 1.0 ml of lyophilized HBV core or HBV e expressing Sindbis vector. Two injections are given one week apart. Seven days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a.

## 2. CHIMPANZEE ADMINISTRATION PROTOCOL

The data generated in the mouse system described above is used to determine the protocol of administration of vector in chimpanzees chronically infected with hepatitis B virus. Based on the induction of HBV-specific CTLs in mice, the subjects in chimpanzee trials will receive four doses of vector encoding core or e antigen at 7 day intervals given in two successively escalating dosage groups. Control subjects will receive a placebo comprised of formulation media. The dosage will be either  $10^7$  or  $10^8$  pfu given in four 1.0 ml injections i.m. on each injection day. Blood samples will be drawn on days 0, 14, 28, 42, 56, 70, and 84 in order to measure serum alanine aminotransferase (ALT) levels, the presence of hepatitis B e antigen, the presence of antibodies directed against the hepatitis B e antigen, serum HBV DNA levels and to assess safety and tolerability of the treatment. The hepatitis B e antigen and antibodies to HB e antigen is detected by Abbott HB e rDNA EIA kit (Abbott Laboratories Diagnostic Division, Chicago, Ill.) and the serum HBV DNA levels is determined by the Chiron bDNA assay. Efficacy of the induction of CTLs against hepatitis B core or e antigen can be determined as in Example 13E 1.c.

Based on the safety and efficacy results from the chimpanzee studies, the dosage and inoculation schedule is determined for administration of the vector to subjects in human trials. These subjects are monitored for serum ALT levels, presence of HBV e antigen, the presence of antibodies directed against the HBV e antigen and serum HBV DNA levels essentially as described above. Induction of human CTLs against hepatitis B core or e antigen is determined as in Example 13E 1.c.

## G. GENERATION OF ELVIS VECTOR CONSTRUCTS WHICH EXPRESS HBV ANTIGENS FOR THE INDUCTION OF AN IMMUNE RESPONSE

## 1. CONSTRUCTION OF ELVIS VECTORS EXPRESSING HBV-C. HBV CORE AND HBV X

Construction of an ELVIS vector expressing the HBV e antigen is accomplished by digesting the SK<sup>+</sup>HB e-c plasmid with Xho I and Not I to release the cDNA fragment encoding HBV e-c sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN<sup>™</sup>, and inserted into pVGELVIS-SINBV-linker vector, previously prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBe.

The HBcore PCR product described previously is digested with Xho I and Cla I, isolated by agarose gel electrophoresis, purified using GENECLEAN<sup>™</sup>, and ligated into SK<sup>+</sup>II (Bluescript, Stratagene, Calif.) digested with Xho I and Cla I. This construct is designated SK<sup>+</sup>HBcore. Construction of the ELVIS vector expressing the HBV core sequence is accomplished by digesting the SK<sup>+</sup>HBcore plasmid with Xho I and Not I to release the cDNA fragment encoding HBVcore sequences. The fragment is then isolated

by agarose gel electrophoresis, purified using GENECLEAN<sup>™</sup>, and inserted into pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBcore.

Construction of the ELVIS vector expressing the HBV-X antigen sequence is accomplished by digesting the plasmid SK-X Ag with Xho I and Not I to release the cDNA fragment encoding HBV-X sequences. The fragment is then isolated by agarose gel electrophoresis, purified using GENECLEAN<sup>™</sup>, and inserted into the pVGELVIS-SINBV-linker vector, prepared by digestion with Xho I and Not I. This construct is designated pVGELVIS-HBX.

Any of the above three constructs can be used for selecting vector infected cells for the generation of HBV specific CTL targets in the following sections.

## 2. EXPRESSION OF TRANSFECTED CELLS WITH ELVIS VECTORS

The pVGELVIS-HBe plasmid DNA is isolated and purified, and 2 ug of pVGELVIS-HBe DNA is complexed with 10 ul of LIPOFECTAMINE<sup>™</sup> and transfected into  $2 \times 10^5$  BHK cells contained in 35 mm petri plates. Two days post-transfection, supernatants and whole cell lysates were collected and an ELISA assay (see below) was used to determine the amount of expressed HBV-e antigen.

Cell lysates from cells infected by any of the sibling pVGELVIS-HBe vectors transfected, are made by washing  $1.0 \times 10^6$  cultured cells with PBS, resuspending the cells to a total volume of 600 ul in PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350 (Fisher, Pittsburgh, Pa.) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

Core antigen and precore antigen in cell lysates and secreted e antigen in culture supernatant are assayed using the Abbott HBe, rDNA EIA kit (Abbott Laboratories Diagnostic Division, Chicago, Ill.). Another sensitive EIA assay for precore antigen in cell lysates and secreted e antigen in culture supernatant is performed using the Incstar ETI-EB kit (Incstar Corporation, Stillwater, Minn.). A standard curve is generated from dilutions of recombinant hepatitis B core and e antigen obtained from Biogen (Geneva, Switzerland).

As shown in FIG. 19, using these procedures, approximately 2 ng/ml HBV e antigen is expressed in the cell lysates and also secreted from BHK cells transfected with different clones of the pVGELVISHBe plasmid.

Characterization of the precore/core and e antigens expressed by vector transfected cells is performed by immunoprecipitation followed by Western blot analysis. Specifically, 0.5–1.0 ml of cell lysate in PBS or culture supernatant is mixed with polyclonal rabbit anti-hepatitis B core antigen (DAKO Corporation, Carpinteria, Calif.) bound to protein G-Sepharose (Pharmacia LKB, Uppsala, Sweden) and incubated overnight at 4° C. Samples are washed twice in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA and boiled in sample loading buffer with 0.5% 2-mercaptoethanol. Proteins are first resolved by SDS polyacrylamide gel electrophoresis, and then transferred to Immobilon (Millipore Corp., Bedford, Me.) and probed with the DAKO polyclonal rabbit anti-hepatitis core B antigen, followed by <sup>125</sup>I-protein A.

## 3. TESTING IMMUNE Response

## (a) Administration Protocols

The mouse model system is also used to evaluate the induction of humoral and cell-mediated immune responses following direct administration of ELVIS vector expressing HBV core or e antigen. Briefly, six- to eight-week-old female Balb/c, C57B1/6, C3H/He mice (Charles River, Mass.) and HLA A2.1 transgenic mice (V. Engelhard,

Charlottesville, Va.) are injected intramuscularly (i.m.) with, for example, 50 ug or greater, pVGELVIS-HBcore, pVGELVIS-HBVe or pVGELVIS-HBX vector DNA. Two injections are given one week apart. Seven or fourteen days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13E 1.a. Detection of humoral immune responses in mice is performed essentially as described in Example 13E 2 and detection of T cell proliferation in mice is performed essentially as described in Example 13E 3.

#### Example 14

#### SINDBIS VECTORS EXPRESSING VIRAL PROTEINS FOR INDUCTION OF THE IMMUNE RESPONSE OR FOR BLOCKING VIRUS HOST CELL INTERACTIONS

The following example describes procedures for constructing Sindbis vectors capable of generating an immune response by expressing an HIV viral antigen. Methods are also given to test expression and induction of an immune response.

#### SINDBIS VECTORS USED TO ELICIT AN IMMUNE RESPONSE

##### A. HIV III ENV EXPRESSION VECTOR

A 2.7 Kb Kpn I-Xho I DNA fragment was isolated from the HIV proviral clone BH10-R3 (for sequence, see Ratner et al., *Nature* 313:277, 1985) and a ~400 bp Sal I-Kpn I DNA fragment from ILX<sub>1</sub>E7deltaenv (a Bal31 deletion to nt 5496) was ligated into the Sal I site in the plasmid SK<sup>+</sup>. From this clone, a 3.1 kb env DNA fragment (Xho I-Not I) was purified and ligated into the previously described Sindbis vectors predigested with Xho I and NotI.

##### B. CREATION OF A PRODUCER CELL LINE WHICH EXPRESSES HIV SPECIFIC ANTIGENS

To construct a vector producing cell line that expresses the HIV IIIB env derived from the vector described above, in vitro transcribed RNA transcripts are transfected in a Sindbis packaging cell line (Example 7). Specifically, the Sindbis RNA vector molecules are initially produced by using a SP6 in vitro transcribed RNA polymerase system used to transcribe from a cDNA Sindbis vector clone encoding the HIV specific sequences. The generated in vitro RNA vector products, are then transfected into a Sindbis packaging or hopping cell line which leads to the transient production of infectious vector particles within 24 hours. These vector particles are then collected from the supernatants of the cell line cultures and then filtered through a 0.45 micron filter to avoid cellular contamination. The filtered supernatants are then used to infect a fresh monolayer of Sindbis packaging cells. Within 24 hours of infection, Sindbis vector particles are produced containing positive stranded Sindbis recombinant RNA encoding Sindbis non-structural proteins and HIV specific sequences.

An alternative configuration of a Sindbis HIV IIIB env vector is a promoter driven cDNA Sindbis construct containing a selectable marker. In this configuration the above-described Xho I to NotI fragment containing the specific HIV IIIB env sequence is placed in a similar cDNA Sindbis vector driven by a constitutive promoter in place of a bacteriophage polymerase recognition sequence. Using this configuration, the expression vector plasmids are transfected into the packaging cell line and selected for the drug resistance gene 24 to 48 hour post-transfection. Resistant

colonies are then pooled 14 days later (dependent on the selection marker used) and diluted and cloned. Several dilution clones are then propagated, and assayed for highest vector titer. The highest titer clones are then expanded and stored frozen. The stored clones are tested for HIV specific protein production and immune response induction.

#### C. TESTING FOR HIV SPECIFIC PROTEIN PRODUCTION AND AN IMMUNE RESPONSE

Cell lysates from the Sindbis HIV producer cell line are tested for HIV specific protein production by Western blot analysis. To test the ability of the vector to transfer expression in vitro, BHK-21 cells are infected with filtered supernatant containing viral vector and assayed by Western blot analysis 24 hours post infection. Once protein expression has been verified in vivo mouse and primate studies can be performed to demonstrate the ability of syngeneic cells expressing a foreign antigen after vector treatment to: (a) elicit a CTL response in mice by injecting either infected syngeneic cells or preparations of infectious vector; (b) elicit CTL responses in a human in vitro culture system; (c) to infect human, chimpanzee and macaque cells, including primary cells, so that these can be used to elicit CTL responses and can serve as targets in CTL assays; (d) map immune response epitopes; and (e) elicit and measure CTL responses to other non-HIV antigens such as mouse CMV (MCMV).

#### 1. IMMUNE RESPONSE TO SINDBIS VIRAL VECTOR-ENCODED ANTIGENS

To test the immune response elicited from a cell line transduced with a Sindbis HIV IIIB env vector, a murine tumor cell line (B/C10ME) (H-2<sup>d</sup>) (Patek et al., *Cell Immunol.* 72:113, 1982) is infected with a recombinant Sindbis virus carrying the HIV IIIB vector. The HIV env expressing cell line (B/C10ME-IIIB) was then utilized to stimulate HIV env-specific CTL in syngeneic (i.e., MHC identical) Balb/c (H-2<sup>d</sup>) mice. Mice are immunized by intraperitoneal injection with B/C10ME-IIIB cells (1×10<sup>7</sup> cells) and boosted on day 7-14. (Boosting may not be required.) Responder spleen cell suspensions are prepared from these immunized mice and the cells cultured in vitro for 4 days in the presence of either B/C10ME-IIIB (BCenv) or B/C10ME (BC) mitomycin-C-treated cells at a stimulator:responder cell ratio of 1:50. The effector cells are harvested from these cultures, counted, and mixed with radiolabeled (<sup>51</sup>Cr) target cells (i.e., B/C10MEenv-29 or B/C10ME) at various effector:target (E:T) cell ratios in a standard 4-5 hour <sup>51</sup>Cr-release assay. Following incubation, the microtitre plates are centrifuged, 100 μl culture supernate is removed, and the amount of radiolabel released from lysed cells quantitated in a Beckman gamma spectrometer. Target cell lysis was calculated as: % Target Lysis = (Exp CPM - SR CPM) / (MR CPM - SR CPM) × 100, where experimental counts per minute (Exp CPM) represents effectors plus targets; spontaneous release (SR) CPM represents targets alone; and maximum release (MR) CPM represents targets in the presence of 1M HCl.

#### 2. STIMULATION OF AN IMMUNE RESPONSE IN MICE BY DIRECT INJECTION OF RECOMBINANT SINDBIS VECTOR

Experiments are performed to evaluate the ability of recombinant Sindbis viral vectors to induce expression of HIV envelope proteins following direct injection in mice. Approximately 10<sup>4</sup> to 10<sup>5</sup> (pfu) of recombinant Sindbis virus carrying the HIV IIIB env vector construct are injected twice (2×) at 3-week intervals either by the intraperitoneal (i.p.) or intramuscular (i.m.) route. This amount of Sindbis virus is determined to be less than the amount considered to



stimulate an immune response. Spleen cells are prepared for CTL approximately 7 to 14 days after the second injection of vector.

#### D. BLOCKING AGENTS DERIVED FROM VIRAL PROTEIN ANALOGUES EXPRESSED FROM RECOMBINANT SINDBIS VECTORS

Many infectious diseases, cancers, autoimmune diseases, and other diseases involve the interaction of viral particles with cells, cells with cells, or cells with factors. In viral infections, viruses commonly enter cells via receptors on the surface of susceptible cells. In cancers, cells may respond inappropriately or not at all to signals from other cells or factors. In autoimmune disease, there is inappropriate recognition of "self" markers. These interactions may be blocked by producing an analogue to either of the partners in an interaction, *in vivo*.

This blocking action may occur intracellularly, on the cell membrane, or extracellularly. The blocking action of a viral or, in particular, a Sindbis vector carrying a gene for a blocking agent, can be mediated either from inside a susceptible cell or by secreting a version of the blocking protein to locally block the pathogenic interaction.

In the case of HIV, the two agents of interaction are the gp 120/gp 41 envelope protein and the CD4 receptor molecule. Thus, an appropriate blocker would be a vector construct expressing either an HIV env analogue that blocks HIV entry without causing pathogenic effects, or a CD4 receptor analogue. The CD4 analogue would be secreted and would function to protect neighboring cells, while the gp 120/gp 41 is secreted or produced only intracellularly so as to protect only the vector-containing cell. It may be advantageous to add human immunoglobulin heavy chains or other components to CD4 in order to enhance stability or complement lysis. Delivery of a Sindbis vector encoding such a hybrid-soluble CD4 to a host results in a continuous supply of a stable hybrid molecule.

Vector particles leading to expression of HIV env analogues may also be constructed as described above. It will be evident to one skilled in the art which portions are capable of blocking virus adsorption without overt pathogenic side effects (Willey et al., *J. Virol.* 62:139, 1988; Fisher et al., *Science* 233:655, 1986).

#### Example 15

##### A. CONSTRUCTION OF FIV ENV/REV/RRE SINDBIS VECTOR FOR THE INDUCTION OF AN IMMUNE RESPONSE

Sequences encoding the FIV env/rev/RRE gene are amplified and isolated from plasmid pFIV-14-Petaluma (NIH Research and Reference Reagent Program, Maryland) using the following primers: The sense primer sequence has two consecutive Xho I restriction sites that are placed at the 5' end at position 6020 of clone 34F10 (Talbot et al., *PNAS* 86:5743-5747, 1989): (SEQ. ID NO.105)

5'-3': CC CTC GAG CTC GAG GGG TCA CTG AGA  
AAC TAG AAA AAG AAT TAG

The antisense primer sequence is complementary to a sequence at position 9387 of clone 34F10. The 5' end of the primer has a Not I site (SEQ.ID NO.106)

5'-3': CC GCG GCC GC GTA TCT GTG GGA GCC TCA  
AGG GAG AAC

The PCR product is then placed in the pBluescript KSII+ plasmid (Stratagene, Calif.) and verified by DNA sequencing. This construct is designated pBluescript KSII+ FIV env/rev/RRE. The Xho I-Not I fragment is then excised and inserted into the Sindbis backbone.

Construction of a Sindbis vector expressing the FIV env/rev/RRE sequence is accomplished by digesting the

SK<sup>+</sup>FIV env/rev/RRE plasmid with Xho I and Not I restriction enzyme sites to release the cDNA fragment encoding FIV env/rev/RRE sequences. The fragment is then isolated by agarose gel electrophoresis, purified by GENECLAN<sup>TM</sup> and inserted into the desired Sindbis vector backbone, prepared by digestion with Xho I and Not I. The Sindbis vectors described in Example 3, are suitable for the insertion of the FIV env/rev/RRE sequences. Such Sindbis vectors include pKSSINBV, pKSSINd1JRsjrc, pKSSINd1JRsjrPC, pKSSIN1JRsjrNP(7582-760 1) and pKSSINd1JRsexjr.

The above Sindbis FIV env/rev/RRE expressing vectors may also be modified to coexpress a selectable drug resistance marker dependent on the requirements of the experiment or treatment of the vector infected cells. Any of the above Sindbis FIV env/rev/RRE expression vectors described may also be designed to coexpress for G418 resistance. This is accomplished by incorporating an internal ribosomal entry site (Example 5) followed by the bacterial neomycin phosphotransferase gene placed 3' of the FIV env/rev/RRE coding sequences and 5' of the terminal 3' end of the vector using the multiple cloning site of the vector. These G418 resistant vector constructs can be used for selecting vector infected cells for the generation of FIV env/rev/RRE specific CTL targets in the following sections.

##### B. INFECTION OF FELINE CELLS WITH SINDBIS VECTOR EXPRESSING FIV ENV/REV/RRE

The feline kidney cell line (CRFK) is grown in DMEM containing 10% FBS. CRFK cells are infected with the Sindbis vector as described in Examples 3 and 7, and used to show vector expression in feline cells using Western blot analysis.

##### C. EXPRESSION OF INFECTED CELLS

Cell lysates from cells infected by any of the FIV env/rev/RRE expressing vectors are made by washing  $1.0 \times 10^7$  cultured cells with PBS, resuspending the cells to a total volume of 600  $\mu$ l in PBS, and sonicating for two 5-second periods at a setting of 30 in a Branson sonicator, Model 350 (Fisher, Pittsburgh, Pa.) or by freeze thawing three times. Lysates are clarified by centrifugation at 10,000 rpm for 5 minutes.

Proteins are separated according to their molecular weight (MW) by means of SDS polyacrylamide gel electrophoresis. Proteins are then transferred from the gel to a IPVH Immobilon-P membrane (Millipore Corp., Bedford, Mass.). The Hoefer HSI TTE transfer apparatus (Hoefer Scientific Instruments, Calif.) is used to transfer proteins from the gel to the membrane. The membrane is then probed with either CE4-13B1 or CE3-8, monoclonal antibodies directed against FIV env gp100. The bound antibody is detected using <sup>125</sup>I-labeled protein A, which allows visualization of the transduced protein by autoradiography.

##### D. TESTING CELLULAR IMMUNE RESPONSE

###### 1. INBRED MICE

Six- to eight-week-old female Balb/c (H-2d), C57B1/6 (H-2b) and C3H/He (H-2k) mice (Charles River, Mass.) are injected twice intraperitoneally (i.p.) at 1 week intervals with  $1 \times 10^6$  pfu of Sindbis FIV env/rev/RRE vector. Animals are sacrificed 7 days later and the splenocytes ( $3 \times 10^6$ /ml) cultured *in vitro* with their respective irradiated ( $10,000$  rads) retroviral vector transduced syngeneic cells (WO 94/06921) ( $6 \times 10^4$ /ml) in T-25 flasks (Corning, Corning, N.Y.). These transduced cells include the murine fibroblast cell lines BC10ME (H-2d) (ATCC No. TIB85), B16 (H-2b) and L-M(TK-) (H-2k) (ATCC No. CCL1.3). These cell lines are grown in DMEM containing 4500 mg/L glucose, 584 mg/L L-glutamine (Irvine Scientific, Santa Ana, Calif.) and



10% FBS (Gemini, Calabasas, Calif.). Culture medium consists of RPMI 1640, 5% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 50 g/ml gentamycin and  $10^{-5}$  M 2-mercaptoethanol (Sigma, St. Louis, Mo.). Effector cells are harvested 4–7 days later and tested using various effector:target cell ratios in 96 well microtiter plates (Corning, Corning, N.Y.) in a standard chromium release assay. Targets are the retroviral vector transduced syngeneic cells (WO 94/06921) whereas the non-transduced syngeneic cell lines are used as negative controls. CTL targets may also be generated from infecting syngeneic cells with the Sindbis FIV env/rev/RRE vector coexpressing the G418 resistance marker. Infected cells are then selected using 800 ug/ml G418 for two weeks. Specifically,  $\text{Na}_2^{51}\text{CrO}_4$ -labeled (Amersham, Arlington Heights, Ill.) (100 uCi, 1 hour at 37° C.) target cells ( $1 \times 10^4$  cells/well) are mixed with effector cells at various effector to target cell ratios in a final volume of 200  $\mu\text{l}$ . Following incubation, 100 ml of culture medium is removed and analyzed in a Beckman gamma spectrometer (Beckman, Dallas, Tex.). Spontaneous release (SR) is determined as CPM from targets plus medium and maximum release (MR) is determined as CPM from targets plus 1M HCl. Percent target cell lysis is calculated as:  $[(\text{Effector cell} + \text{target CPM}) - (\text{SR})] / (\text{MR}) - (\text{SR}) \times 100$ . Spontaneous release values of targets are typically 10%–20% of the MR.

For certain CTL assays, the effectors may be in vitro stimulated multiple times, for example, on day 8–12 after the primary in vitro stimulation. More specifically,  $10^7$  effector cells are mixed with  $6 \times 10^5$  irradiated (10,000 rads) stimulator cells, and  $2 \times 10^7$  irradiated (3,000 rads) “filler” cells (prepared as described below) in 10 ml of “complete” RPMI medium. (RPMI containing: 5% heat inactivated Fetal Bovine Serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1X non essential amino acids, and  $5 \times 10^{-5}$  M 2-mercaptoethanol). Stimulator cells for in vitro stimulation of effector cells are generated from irradiated retroviral vector transduced syngeneic cells. “Filler” cells are prepared from naive syngeneic mouse spleen cells resuspended in RPMI, irradiated with 3,000 rads at room temperature. Splenocytes are washed with RPMI, centrifuged at 3,000 rpm for 5 minutes at room temperature, and the pellet is resuspended in RPMI. The resuspended cells are treated with 1.0 ml tris-ammonium chloride (100 ml of 0.17 M tris base, pH 7.65, plus 900 ml of 0.155 M  $\text{NH}_4\text{Cl}$ ; final solution is adjusted to a pH of 7.2) at 37° C. for 3–5 minutes. The secondary in vitro restimulation is then cultured for 5–7 days before testing in a CTL assay. Any subsequent restimulations are cultured as described above with the addition of 2–10 U of recombinant human IL-2 (200 U/ml, catalog #799068, Boehringer Mannheim, W. Germany).

## 2. FELINES

Since the vectors are to be utilized for treating felines, an assay demonstrating immunological efficacy in felines is needed. The following is a description of the generation of the autologous T-cell lines needed for restimulator and target cells for the standard  $^{51}\text{Cr}$  release assay (Brown et al., *J. Vir.* 65:3359–3364, 1991). Briefly, peripheral blood mononuclear cells (PBMC) are obtained following venipuncture and Ficoll-sodium diatrizoate (Histopaque-1077; Sigma, St. Louis, Mo.) density gradient centrifugation. These PBMCs are stimulated by 5 ug/ml concanavalin A (Con A, Sigma) for three days, and maintenance in medium containing 25 U/ml human recombinant interleukin-2 (IL-2) (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and 10% bovine T-cell growth factor (TCGF). Cells are seeded into round bottom 96-well microtiter plates at an average of 1 or 0.3 cells per well with  $5 \times 10^4$  irradiated (3,000 rads) autolo-

gous PBMC, 10% bovine TCGF, and 25 U/ml of IL-2 in a final volume of 200  $\mu\text{l}$  of complete RPMI. Complete RPMI consist of RPMI 1640 medium containing 10% FBS, 2mM L-glutamine,  $5 \times 10^{-5}$  M 2-mercaptoethanol, and 50 ug of gentamycin per ml. Clones are expanded sequentially to 48-well and 24-well plates. After several weeks, cells are transduced with retroviral vectors expressing FIV env/rev genes (WO 94/06921), and selected with G418. Expression of these cell lines are monitored by Western blot analysis as in Example 15C. Cell lines expressing high levels of the desired protein function as stimulators and targets in a standard  $^{51}\text{Cr}$  release assay as in Example 15 D 1. Effector cells are recovered for the CTL assay from the peripheral blood mononuclear cells (PBMC) obtained following venipuncture and Ficoll-sodium diatrizoate density gradient centrifugation.

## E. ADMINISTRATION PROTOCOLS

Six- to eight-week-old female Balb/C, C57B16 or C3H/He mice are injected intramuscularly (i.m.) with 0.1 ml of reconstituted (with sterile deionized, distilled water) or intraperitoneally (i.p.) with 1.0 ml of lyophilized FIV env/rev/RRE expressing Sindbis vector. Two injections are given one week apart. Seven days after the second injection, the animals are sacrificed. Chromium release CTL assays are then performed essentially as described in Example 13 D 1.

Felines are also injected intramuscularly (i.m.) with 0.5 ml of reconstituted (with sterile deionized, distilled water) or intraperitoneally (i.p.) with 2.0 ml of lyophilized FIV env/rev/RRE expressing Sindbis vector. Two injections are given one week apart. Seven days after the second injection, PBMCs are withdrawn for the CTL assay. Chromium release CTL assays are then performed essentially as described in Example 13 D 2.

## Example 16

### TISSUE SPECIFIC EXPRESSION BY ACTIVATION OF DISABLED ALPHAVIRUS

#### VECTORS USING TISSUE SPECIFIC CELLULAR RNA: CONSTRUCTION OF

#### ALPHAVIRUS TUMOR SPECIFIC EXPRESSION VECTORS FOR THE TREATMENT OF

#### COLORECTAL CANCER

#### A. CONSTRUCTION OF A RECOMBINANT SINDBIS VECTOR (SIN-CEA) DEPENDENT ON THE EXPRESSION OF THE CEA TUMOR MARKER

As described previously and shown diagrammatically in FIG. 20, the disabled junction loop out model is constructed with the junction region of the vector flanked by inverted repeat sequences which are homologous to the RNA of choice. In this example, sequences from the CEA tumor antigen cDNA (Beauchemin et al., *Molec. and Cell. Biol.* 7:3221, 1987) are used in the inverted repeats. To construct a CEA RNA responsive Sindbis vector, the junction region is preceded by two CEA anti-sense sequence domains (A<sup>1</sup> and B<sup>1</sup>) separated by a six base pair hinge domain. A single twenty base pair CEA sense sequence (A2), which is complementary to A1, is placed at the 3' end of the junction region. In choosing the correct A1 and B1 antisense sequences, the only two requirements are that they be specific for the targeted RNA sequence and that the antisense sequences hybridize to two RNA sequence domains separated by three nucleotides. This three nucleotide gap will serve as a hinge domain for the polymerase to hop and switch reading strands bridging the non-structural protein

domain of the vector to the junction region of the vector (FIG. 5). To construct such a configuration, two oligonucleotides are synthesized complementing each other to create a fragment insert containing convenient restriction enzyme sites at the extreme 5' and 3' ends. The oligonucleotide fragment insert is then ligated into the Sindbis vector between the disabled junction region and the multiple cloning sites of the Sindbis vector. The sense oligonucleotide strand, from 5' to 3', should contain an Apa I restriction site, followed by the A1 anti-sense domain, a six bp hinge domain, a B1 anti-sense domain, a synthetic junction region domain, and the A2 sense domain, followed by a Xho I restriction enzyme site. The following oligonucleotide sequence is used to design a CEA RNA responsive Sindbis vector. The nucleotide number sequence is obtained from Beauchemin et al., *Molec. and Cell Biol.* 7:3221, 1987.

#### 5'-3' CEA sense strand:

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          CEA 618                                CEA 589
          *-----*
    Apa I  GC G GGC CCT GT G ACA T TG AAT AGA GT G AGG G TC CTG
    TTG GG (SEQ. ID NO.107)

          CEA 651                                CEA 622
          *-----*
    A AAG G TT TCA CAT TT G TAG C TT GCT GTG TC A TTG C GA TCT
    CTA CG (SEQ. ID NO.108)

          CEA 599                                CEA 618
          *-----*
    Junction Core  G TGG T CC TAA ATA GT T CAC CT ATT CAA TG T CAC A CT CGA
    GCC GG (SEQ. ID NO.109)
  
```

The 5'-3' CEA anti-sense strand is complementary to the above oligonucleotide. After both oligonucleotides are synthesized, the oligonucleotides are mixed together in the presence of 10 mM Mg, heated to 100° C. for 5 minutes and cooled slowly to room temperature. The oligonucleotide pair is then digested with the Apa I and Xho I restriction enzymes, mixed and ligated at a 25:1 molar ratio of insert to plasmid, pCMV-SIN or pMET-SIN predigested with the same enzymes. These constructs are designated pCMV/SIN-CEA and pMET/SIN-CEA, respectively.

#### CONSTRUCTION OF A SIN-CEA VECTOR AND PRODUCER CELL LINE EXPRESSING GAMMA INTERFERON (SIN-CEA/IFN)

The human gamma interferon gene is subcloned from the retroviral vector plasmid pHu-IFN- $\gamma$  (Howard et al., *Ann N.Y. Acad. Sci.* 716:167-187, 1994) by digesting with Xho I and Cla I. The resulting 500 bp fragment containing the coding sequences of  $\gamma$ -IFN is isolated from a 1% agarose gel.

Alternatively, the human  $\gamma$ -IFN cDNA is derived from RNA isolated from PHA-stimulated Jurkat T cells by guanidinium thiocyanate extraction followed by ultracentrifugation through a CsCl gradient. The RNA (Sigma, St. Louis, Mo.) is then reverse-transcribed in vitro and a gene-specific oligonucleotide pair is used to amplify  $\gamma$ -IFN cDNA by polymerase chain reaction using Taq polymerase. The PCR DNA was repaired with T4 DNA polymerase and Klenow and cloned into the Hinc II site of SK<sup>+</sup> plasmid (Stratagene, San Diego, Calif.) treated with CLAP. In the sense orientation, the 5' end of the cDNA is adjacent to the Xho I site of the SK<sup>+</sup> polylinker and the 3' end adjacent to the NotI site. The 512 base pair fragment encoding the human  $\gamma$ -IFN molecule is placed into the Xho I/NotI site of either the pCMV/SIN-CEA or pMET/SIN-CEA vectors. These new plasmids are designated pCMV/SIN-CEA/IFN- $\gamma$  or pMET/SIN-CEA/IFN- $\gamma$ , respectively.

#### B. CONSTRUCTION OF A SIN-CEA VECTOR AND PRODUCER CELL LINE EXPRESSING THYMIDINE KINASE (SIN-CEA/TK)

A PCR amplified product containing the cDNA clone of the herpes simplex thymidine kinase ("HSVTK"), flanked with 5' Xho I and 3' NotI restriction enzyme sites is obtained using the pHS1TK3KB (McKnight et al., *Nuc. Acids Res.* 8:5949, 1980) clone as target DNA. The sequences for the primers used for the PCR amplification are obtained from published sequences (Wagner et al., *PNAS* 78:1442, 1981). The 1,260 base pair amplified product is then digested with Xho I and NotI ligated into the Xho I/NotI site of either the pCMV/SIN-CEA or pMET/SIN-CEA vectors. These new plasmids are designated pCMV/SIN-CEA/HSVTK or pMET/SIN-CEA/HSVTK, respectively.

#### C. CREATION OF CEA RNA DEPENDENT SINDBIS VECTOR PRODUCER CELL LINES

Unlike the previous examples of creating producer cell lines (Example 7), it may be that only a single round of gene transfer into the packaging cell line is possible by vector transfection. Since these vectors will be disabled and prevented in the synthesis of full genomic vectors, re-infection of a fresh layer of Sindbis packaging cell lines will end in an aborted infection since these vectors are now dependent on the presence of the CEA RNA to become active. Higher titers may be achieved by dilution cloning transfecting producer cell lines using the RT-PCR technique.

#### Example 17

#### REPLACEMENT GENE THERAPY USING RECOMBINANT ALPHAVIRUS VECTORS

The following example describes the construction of alphavirus vectors capable of generating a therapeutic protein.

#### A. CONSTRUCTION OF A SINDBIS FACTOR VIII VECTOR

Hemophilia A disease is characterized by the absence of Factor VIII, a blood plasma coagulating factor. Approximately 1 in 20,000 males have hemophilia A in which the disease state is presented as a bleeding disorder, due to the inability of affected individuals to complete the blood clotting cascade.

The treatment of individuals with hemophilia A is replacement with the Factor VIII protein. The only source for human Factor VIII is human plasma. In order to process human plasma for Factor VIII purification, human donor samples are pooled in lots of over 1000 donors. Due to the instability of the Factor VIII protein, the resulting pharmaceutical products are highly impure, with an estimated purity by weight of approximately 0.04%. In addition, there is a serious threat of such infectious diseases as hepatitis B virus and the Human Immunodeficiency Virus, among others,

which contaminate the blood supply and can thus be potentially co-purified with the Factor VIII protein.

The Factor VIII cDNA clone is approximately 8,000 bps. Insertion of the Factor VIII cDNA into pKSSINBV yields a vector/heterologous gene genomic size of approximately 15,830 bps. If the packaging of this large vector RNA into particles is inefficient, the size of the insert can be decreased further by eliminating the "B-domain" of the Factor VIII insert. It has been shown that the Factor VIII B-domain region can be removed from the cDNA without affecting the functionality of the subsequently expressed protein.

A Sindbis-Factor VIII vector is constructed as follows. Factor VIII cDNA is obtained from clone pSP64-VIII, an ATCC clone under the accession number 39812, containing a cDNA encoding the full-length human protein. pSP64-VIII is digested with Sal I, the termini are blunted with T4 DNA polymerase and 50 uM of each dNTP, and the ca. 7700 bp. fragment is electrophoresed in a 1% agarose/TBE gel and purified with GENECLEAN™. The Factor VIII cDNA containing blunt ends is then ligated into pKSI13'SIN (Example 3), prepared by digestion with Hinc II, treated with CIAP, and purified from a 1% agarose gel. This plasmid is known as pF83'SIN.

For insertion of Factor VIII into the various Sindbis vectors described in Example 3, plasmid pF83'SIN is digested with Xho I and a limited Sac I digest, and the resulting 7,850 bp fragment is isolated from a 0.7% agarose/TBE gel. This Factor VIII-3'SIN fragment is then inserted into each of the vectors listed below. Prior to insertion of this fragment the plasmids are prepared by digestion with Xho I and Sac I, treated with CIAP, isolated by 1% agarose/TBE gel electrophoresis, and purified with GENECLEAN™:

Vector	Functional Junction Region (+/-)
pKSSINBV	+
pKSSINd1JRsjrc	+
pKSSINd1JRsjrPC	+
pKSSINd1JRsjrNP(7,582-7,601)	+
pKSSINd1JRsejrf8	+

Following insertion of the Factor VIII cDNA, these vectors are designated:

pKSSINBVF8  
pKSSINd1JRsjrcF8  
pKSSINd1JRsjrPCF8  
pKSSINd1JRsjrNP(7,582-7,601)F8  
pKSSINd1JRsejrf8

respectively. Packaging of the Factor VIII cDNA containing vectors is accomplished by the transfection of packaging cell lines (described in Example 7) with in vitro transcribed vector/Factor VIII RNA. The efficiency of packaging is determined by measuring the level of Factor VIII expression in cells infected with the packaged vector and compared to similar experiments performed with the pKSSIN-luc vector described in Example 3.

#### B. CONSTRUCTION OF A GLUCOCEREBROSIDASE SINDBIS VECTOR

Gaucher disease is a genetic disorder that is characterized by the deficiency of the enzyme glucocerebrosidase. This enzyme deficiency leads to the accumulation of glucocerebroside in the lysosomes of all cells in the body. However, the disease phenotype is manifested only in the macrophages, except in the very rare neuropathic forms of the disease. The disease usually leads to enlargement of the liver and spleen and lesions in the bones. (For a review, see

*Science* 256:794, 1992, and *The Metabolic Basis of Inherited Disease*, 6th ed., Scriver et al., vol. 2, p. 1677.)

A glucocerebrosidase Sindbis vector is constructed as follows. Briefly, a glucocerebrosidase (GC) cDNA clone containing a Xho the cDNA coding sequence site 5' and 3' of the cDNA coding sequence is first generated. The clone is generated by digesting pMFG-GC (Ohashi et al., *PNAS* 89:11332, 1992) with Nco I, blunt-ending the termini with T4 DNA polymerase and dNTPs, ligating with Xho I linkers, and purifying the GC gene from a 1% agarose gel. The GC fragment is subsequently digested with Xho I and ligated with the desired Sindbis vector (for example, pKSSINBV) that has also been digested with Xho I. Packaging of the Sindbis-glucocerebrosidase vector is accomplished by introduction of vector RNA (for example, transfection of in vitro transcribed RNA) into any of the packaging cell lines described in Example 7.

Both the Sindbis Factor VIII and the Sindbis Glucocerebrosidase vectors are also readily convertible to plasmid DNA based-vectors which initiate vector replication and heterologous gene expression for use in direct delivery or the establishment of vector producer cell lines (see Examples 3 and 7).

#### Example 18

##### INHIBITION OF HUMAN PAPILLOMA VIRUS PATHOGENICITY BY SEQUENCE-SPECIFIC ANTISENSE OR RIBOZYME MOLECULES EXPRESSED FROM SINDBIS VIRUS VECTORS

To date, more than sixty types of human papilloma viruses (HPV), which have a pronounced tropism for cells of epithelial origin, have been isolated and characterized. Among the HPV group are a substantial number of types which infect the human anogenital tract. This group of HPVs can be further subdivided into types which are associated with benign or with malignant proliferation of the anogenital tract.

There are between 13,000 and 20,000 cervical cancer deaths per year in the U.S. In developing countries, cervical cancer is the most frequent malignancy, and in developed countries cervical cancer ranks behind breast, lung, uterus, and ovarian cancers. One statistic which especially supports the notion that anogenital proliferation is a growing health problem is that medical consultations for genital warts increased from 169,000 in 1966 to greater than 2 million in 1988.

Several lines of evidence exist which link HPV to the pathogenesis of cervical proliferative disease. A distinct subset of types, so called 'low risk HPVs', are associated with benign proliferative states of the cervix (e.g., HPV 6, 11, 43, 44), while another subset of types, the 'high risk HPVs', are associated with lesions which may progress to the malignant state (e.g., HPV 16, 18, 31, 33, 35, etc.). Approximately 95% of cervical tumors contain HPV, with HPV type 16 or 18 DNA being found in about 70% of them.

The frequency of HPV in the young sexually active female population appears to be quite high. Indeed, in a recent study of 454 college women, 213, or 46% were HPV positive. Among the HPV positive group, 3% were HPV 6/11 positive, and 14% were HPV 16/18 positive. Of these 454 women, 33 (7.3%) had abnormal cervical proliferation, as determined by cytology.

With regard to the design of antisense and ribozyme therapeutic agents targeted to HPV, there are important parameters to consider relating to the HPV types to target (i.e., types associated with condyloma acuminatum or types associated with malignant cervical proliferation) and HPV expressed genes to target, including but not limited to, HPV genes E2, E6, or E7.

In general, the expression of HPV genes is defined temporally in two phases, early (E) genes expressed prior to viral DNA replication, and late (L) genes expressed after viral DNA replication. There are 7 early enzymatic HPV genes, and 2 late structural HPV genes.

Based on the discussion presented above, antisense/ribozyme therapeutics directed towards the HPV 6/11 groups may be constructed which target the viral E2 gene. It seems possible that the E2 gene target may be precarious with regard to the HPV 16/18 group, by a mechanism of driving integration of the virus through inhibition of E2 protein expression. Thus, it seems that the E6/E7 genes in HPV types 16/18 should be targeted directly.

Described below is the construction of antisense and ribozyme therapeutics into Sindbis virus vectors (described in Example 2) specific for HPV type 16 E6 and E7 RNA. Insertion of the HPV antisense and ribozyme moieties is between the Cla I and Xba I sites of the Sindbis vector.

#### A. CONSTRUCTION OF AN HPV 16 E6/E7 ANTISENSE THERAPEUTIC

The HPV 16 viral genomic clone, pHPV-16 (ATCC number 45113) is used as a template in a PCR reaction for the amplification of specific sequences from the viral E6/E7 genes. The HPV 16 antisense moiety is first inserted into the plasmid vector pKSI<sup>+</sup>; removal of the antisense therapeutic from the plasmid vector and insertion into the various Sindbis vector backbones is accomplished via the unique antisense moiety terminal Cla I and Xba I restriction endonuclease sites. Amplification of a portion of the HPV 16 E6/E7 genes is accomplished with the primer pair shown below:

Forward primer (buffer sequence/Xba I site/HPV 16 nucleotides 201–222):

TATATTCTAGAGCAAGCAACAGTTACTGCGACG (SEQ.ID NO.110)

Reverse primer (buffer sequence/Cla I site/HPV 16 nucleotides 759–738):

TATATATCGATCCGAAGCGTAGAGTCACACTIG (SEQ.ID NO.111)

In addition to the HPV 16 E6/E7 complementary sequences, both primers contain a five nucleotide 'buffer sequences' at their 5' ends for efficient enzyme digestion of the PCR amplicon products. Generation of the HPV 16 amplicon with the primers shown above is accomplished with the PCR protocol described in Example 4. It has been shown previously that the E6/E7 mRNA in infected cervical epithelia is present in three forms, unspliced and two spliced alternatives (E6\* and E6\*\*), one in which nucleotides 226–525 of E6 are not present in the mature message (Smotkin et al., *J. Virol* 63:1441–1447, 1989). The region of complementarity between the antisense moiety described here and the HPV 16 genome is viral nucleotides 201–759. Thus the antisense moiety will be able to bind to and inhibit the translation of the E6/E7 unspliced message and the spliced E6\* and E6\*\* spliced messages.

The HPV 16 E6/E7 580 bp amplicon product is first purified with GENECLAN<sup>TM</sup>, digested with the restriction enzymes Cla I and Xba I, and electrophoresed on a 1% agarose/TBE gel. The 568 bp band is then excised from the gel, the DNA purified with GENECLAN<sup>TM</sup> and ligated into the pKSI<sup>+</sup> plasmid prepared by digestion with Cla I and Xba I, treatment with CIAP, and treatment with GENECLAN<sup>TM</sup>. This plasmid is known as pKSaHPV16E6/E7.

#### B. CONSTRUCTION OF HPV 16 E6/E7 HAIRPIN RIBOZYME THERAPEUTICS

In order to efficiently inhibit the expression of HPV 16 E6 and E7 proteins, a hairpin ribozyme (HRBZ) with target

specificities to E6 mRNA is constructed. The HPV 16 ribozyme moiety is first inserted into the plasmid vector pKSI<sup>+</sup>; removal of the ribozyme therapeutic from the plasmid vector and insertion into the various Sindbis vector backbones is accomplished via the unique ribozyme moiety terminal Cla I and Xba I restriction endonuclease sites.

The HRBZ is homologous to the HPV 16 E6 RNA (nts 414–431) shown below:

TTAACTGTCAAAAGCCAC (SEQ.ID NO. 112)

The HRBZ is designed to cleave after the T residue in the TCTC hairpin ribozyme loop 5 substrate motif, shown underlined above. Following cleavage, the HRBZ is recycled and able to hybridize to, and cleave, another unspliced E6/E7 mRNA or the E6\* spliced mRNA molecule.

Double-stranded HRBZ as defined previously (Hampel et al., *Nucleic Acids Research* 18:299–304, 1990), containing a 4 base 'tetraloop' 3 and an extended helix 4, with specificity for the HPV 16 E6 RNA shown above, is chemically synthesized and includes both the 5' and 3' ends, respectively, Cla I and Xba I sites. The sequence of the chemically synthesized HPV 16 E6 HRBZ strands are shown below:

HPV 16 E6 HRBZ, sense strand (5'→3'):

5'-CGATGTGGCTTTTAGATGTAAACCAGAGAA-  
A C A C A C G G A C T T C G G T C  
CGTGGTATATTAGCTGGTAT-3' (SEQ.ID NO. 1 13)

HPV 16 E6 HRBZ, antisense strand (5'→3'):

5'-CTAGATACCAGCTAATATACCACGGACCGAA-  
G T C C G T G T G T T C T C T G G  
TTTAACATCTAAAAGCCACAT-3' (SEQ.ID NO.  
114)

In order to form the double-stranded HPV 16 E6 specific HRBZ with Cla I and Xba I cohesive ends, equal amounts of the oligonucleotides are mixed together in 10 mM Mg<sup>2+</sup>, heated at 95° C. for 5 minutes, then cooled slowly to room temperature to allow the strands to anneal.

The double-stranded HPV 16 E6 HRBZ with Cla I and Xba I cohesive ends is first ligated into the pKSI<sup>+</sup> plasmid vector, prepared by digestion with Cla I and Xba I, treatment with CIAP, and treatment with GENECLAN<sup>TM</sup>. This plasmid is known as pKSHPV16E6HRBZ.

The HPV 16 antisense and hairpin ribozyme moieties are liberated from their plasmid vectors, pKSaHPV16E6/E7 and pKSHPV16E6HRBZ, respectively, by digestion with Cla I and Xba I, purification by agarose electrophoresis and GENECLAN<sup>TM</sup>, and insertion into the desired vector backbone, prepared by digestion with Cla I and Xba I, and treatment with CIAP. Several possible Sindbis vectors some of which are shown below, and whose detailed construction is described in Example 2, are suitable for the insertion of the HPV 16 antisense and ribozyme therapeutic moieties:

Vector	Functional Junction Region (+/-)
pKSSINBV	+
pKSSINBVd1JR	+
pKSSINd1JRsjr	+
pKSSINd1JRsjrPC	+
pKSSINd1JRsjrNP(7582–7601)	+
pKSSINd1JRsjr	+

Since the antisense and ribozyme therapeutic operate at the level of RNA, it is not necessary that the vectors containing these moieties contain a functional junction region. That is, translation of the region corresponding to the Sindbis structural proteins occurs only from subgenomic

RNA. However, because translation of the antisense and hairpin ribozyme therapeutic is not an issue, these moieties will exert their affect from the level of positive stranded Sindbis genomic vector RNA.

On the other hand, it may be desired to administer repeated doses to an individual; thus the antisense and hairpin palliative would be inserted downstream of the adenovirus E3 or human cytomegalovirus H301 genes, which down-regulate the expression of MHC class I molecules in infected cells. Insertion of the antisense and hairpin palliatives is accomplished in the vectors from Examples 3 and 4 shown below, between the Cla I and Xba I sites:

Vector	Functional Junction Region (+/-)
pKSSINd1JRsjrcAdE3	+
pKSSINd1JRsjrcH301	+

Subgenomic mRNA is synthesized in these vectors, which serves as a translational template for the Ad E3 and CMV H301 genes. Thus, in these constructions, functional HPV 16 antisense and hairpin ribozyme palliatives will be present on the levels of both subgenomic and positive stranded genomic Sindbis vector RNA.

Further, the HPV 16 antisense and hairpin ribozyme palliatives can be inserted downstream of a heterologous gene inserted into the described Sindbis vectors. For example, one could insert the HPV 16 antisense and hairpin ribozyme palliatives downstream of a heterologous gene coding for an immunogenic epitope of HPV 16 from, for example, the E6/E7 or L1 proteins. In these vectors, it would not be desired to include the immunoregulatory Ad E3 or CMV H301 genes.

Expression of the E6/E7 genes during infection with both the high- and low-risk HPV groups is required for proliferation of the cervical epithelium. The HPV E7 protein from all HPV types tested forms a complex with the retinoblastoma protein, and the E6 protein from HPV types 16 and 18 associates with and degrades the cellular p53 protein. The p53 and retinoblastoma cellular gene products are involved in the growth control of the cell, and altering the expression or function of these proteins can release the growth control in affected cells. Thus, an antisense or ribozyme therapeutic agent to both HPV groups should either directly or ultimately diminish the expression of one or both of these genes. Expression of the E6/E7 genes is trans-activated by the viral E2 protein. However, by utilizing an alternative splicing strategy, the E2 protein can also act as a trans-repressor. Integration of the oncogenic HPV types occurs in the viral E2 region and abrogates the expression of the E2 protein. Integration by the oncogenic HPV types appears to be a pivotal event in the frank induction and/or maintenance of cervical carcinoma. This event results in the constitutive expression of the E6/E7 genes. In the integrated state, expression of the E6/E7 genes is trans-activated by factors present in infected keratinocytes. The inactivation of the viral E2 control mechanism in response to the cellular keratinocyte factor activation of E6/E7 expression might be a critical event in viral integration.

#### Example 19

#### INHIBITION OF HUMAN INTERFERON A EXPRESSION IN INFECTED CELLS BY SEQUENCE-SPECIFIC RIBOZYME MOLECULES EXPRESSED FROM SINDBIS VIRUS VECTORS

Interferons (IFNs) comprise a family of small proteins which effect a wide range of biological activities in the

mammalian cell, including the expression of MHC antigens, the expression of several genes which modulate cell growth control, and the resistance to viral infections (Pestka et al., *Ann. Rev. Biochem.* 56:727-777, 1987). Of the three classes of IFNs,  $\alpha$ ,  $\beta$ , and  $\gamma$ -IFN,  $\alpha$ -IFN, or leukocyte interferon, has a key role in limiting viral replication in the infected cell.

The antiviral effects of IFN- $\alpha$  are associated with the induction of two cellular enzymes which inhibit the viral lifecycle in the infected cell. One enzyme is a double-stranded RNA dependent 68-kDa protein kinase that catalyzes the phosphorylation of the  $\alpha$  subunit of the protein synthesis initiation factor eIF-2. The second enzyme induced by IFN- is 2',5'-oligoadenylate synthetase (2',5'-OAS), which in the presence of double-stranded RNA activates the latent endonuclease, RNase L, which is responsible for degradation of viral and cellular RNAs (Johnston and Torrence, *Interferons* 3:189-298, Friedman (ed.), Elsevier Science Publishers, B.V., Amsterdam, 1984).

Because their replication strategy includes a double-stranded RNA intermediate, the RNA viruses in particular are strong inducers of interferon. With regard to Sindbis virus, double-stranded RNA molecules are present during the replication of both positive- and negative-stranded genome length molecules, and during the transcription of subgenomic mRNA. It has been demonstrated that infection of cells with Sindbis virus results in the induction of interferon (Saito, *J. Interferon Res.* 9:23-24, 1989).

In applications where extended expression of the therapeutic palliative is desired, expression of IFN in the infected cell is inhibited by inclusion of a hairpin ribozyme with specificity for IFN- $\alpha$  mRNA in the Sindbis vector. Inhibition of IFN- expression thus mitigates induction of the cascade of cellular proteins, including the eIF-2 protein kinase and 2',5'-OAS, which inhibit the extent to which virus can replicate in the infected cell. Prolonged expression of the therapeutic palliative without induction of an immune response targeted towards the vector infected cell is desired in all applications other than antigen presentation and includes, for example, systemic protein production, antisense and ribozyme, and accessory molecules.

#### A. CONSTRUCTION OF A HAIRPIN RIBOZYME WITH TARGETED SPECIFICITY FOR INTERFERON A MRNA

In order to efficiently inhibit the expression of interferon a protein in cells infected with Sindbis vectors, a hairpin ribozyme (HRBZ) with target specificity for interferon  $\alpha$  mRNA is constructed. The IFN- $\alpha$  ribozyme moiety is first inserted into the plasmid vector pKSII\* (Stratagene, La Jolla, Calif.); removal of the ribozyme therapeutic from the plasmid vector and insertion into the various Sindbis vector backbones is accomplished via the unique ribozyme moiety terminal Cla I and Xba I restriction endonuclease sites.

The HRBZ is homologous to nucleotides 1026-1041 of the human interferon alpha gene IFN-alpha 4b shown below, and to all IFN- $\alpha$  genes sequenced, including 5, 6, 7, 8, and 14, but not gene 16 (Henco et al., *J. Mol. Biol.* 185:227-260, 1985):

5'-TCT CTG TCC TCC ATG A (SEQ.ID NO. 120)

The HRBZ is designed to cleave after the T residue in the TGTC hairpin ribozyme loop 5 substrate motif, shown underlined above. Following cleavage, the HRBZ is recycled and able to hybridize to, and cleave, another IFN- $\alpha$  mRNA molecule.

Double-stranded HRBZ as defined previously (Hampel et al., *Nucleic Acids Research* 18:299-304, 1990), containing a 4 base tetraloop 3 and an extended helix 4, with specificity for the IFN- $\alpha$  mRNA shown above, is chemically synthesized and includes at the 5' and 3' ends, respectively, Cla I and Xba I sites. The sequence of the chemically synthesized IFN- $\alpha$  HRBZ strands are shown below:

IFN- $\alpha$  HRBZ. sense strand (5' to 3'):

TCG AGT CAT GGA GAG AGG AGA ACC AGA GAA  
ACA CAC GGA CTT CGG TCC GTG GTA TAT TAC  
CTG GAT (SEQ.ID NO. 121)

IFN- $\alpha$  HBRZ. antisense strand (5' to 3'):

CGA TCC AGG TAA TAT ACC ACG GAC CGA AGT  
CCG TGT GTT T CTCTG GTT C TC CTC TCT CCA  
TGA C (SEQ.ID NO. 122)

In order to form the double-stranded IFN- $\alpha$  specific HRBZ with Cla I and Xba I cohesive ends, equal amounts of the oligonucleotides are mixed together in 10 mM Mg<sup>2+</sup>, heated at 95° C. for 5 minutes, then cooled slowly to room temperature to allow the strands to anneal.

The double-stranded IFN- $\alpha$  HRBZ with Cla I and Xba I cohesive ends is first ligated into the pKSIH<sup>+</sup> plasmid vector, prepared by digestion with Cla I and Xba I, treatment with CIAP, and treatment with GENECLEAN™. This plasmid is known as pKSIFN $\alpha$ HRBZ.

The IFN- $\alpha$  hairpin ribozyme moiety is liberated from the pKSIFN $\alpha$ HRBZ plasmid by digestion with Cla I and Xba I, purification by 2% Nu-Sieve/1% agarose electrophoresis and GENECLEAN™, and insertion into the desired vector backbone, prepared by digestion with Cla I and Xba I, and treatment with CIAP. Several possible Sindbis vectors some of which are shown below, and whose detailed construction is described in Examples 2, 3, and 4 are suitable for the insertion of the IFN- $\alpha$  hairpin ribozyme moiety:

Vector	Functional Junction Region (+/-)
pKSSINBV	+
pKSSINBVdUR	-
pKSSINdURsjrc	+
pKSSINdURsjrPC	+
pKSSINdURsjrNP(7582-7601)	+
pKSSINdURsexjr	+
pKSSINdURsjrcAdE3	+
pKSSINdURsjrcH301	+

Since the ribozyme activity operates at the level of RNA, it is not necessary that this region is expressed as a portion subgenomic mRNA. However, when placed downstream of a functional junction region, the level of ribozyme synthesized is much greater and perhaps more effective in cleaving the IFN- $\alpha$  RNA target.

Further, in some applications, for example systemic expression of protein, multiple dose administration to an individual is required. In these applications, prolonged expression of the therapeutic palliative without induction of an immune response targeted towards the vector infected cell is desired. In this configuration, the IFN- $\alpha$ HRBZ moiety could be inserted upstream of the adenovirus E3 or human cytomegalovirus H301 genes, which down-regulate the expression of MHC class I molecules in infected cells. Following the gene which modulates MHC class I expression is, consecutively, an IRES element selected from among the group described in Example 5, and the therapeutic palliative. Ordered insertion of the hairpin ribozyme, Ad E3 or CMV H301, IRES, and heterologous gene of interest components along the multiple cloning sequence located in the vector between the vector junction region and 3' end is accomplished by modification with the appropriate restriction enzyme recognition sites of the component 5' and 3' ends. In these constructions, functional INF- $\alpha$  hairpin ribozyme palliatives will be present at the level of both subgenomic and positive stranded genomic Sindbis vector RNA.

## EX VIVO AND IN VIVO TREATMENT OF HUMAN CANCERS BY ADMINISTRATION OF

### RECOMBINANT ALPHAVIRUS VECTOR PARTICLES OR ALPHAVIRUS PLASMID DNA

#### VECTORS WHICH EXPRESS CYTOKINES, CYTOKINE RECEPTORS, OR DRUG POTENTIATORS

##### A. VECTOR CONSTRUCTIONS

##### 1. GAMMA INTERFERON

Murine gamma interferon is subcloned from the retroviral vector plasmid pMu- $\gamma$ IFN (Howard et al., *Ann. N.Y. Acad. Sci.* 716:167-187, 1994) by digesting with Cla I and making the termini blunt by Klenow enzyme and dNTPs. After heat inactivation of the Klenow enzyme, the vector is digested with Xho I. The resulting 800 bp fragment containing the coding sequences of gamma interferon is isolated from a 1% agarose gel. pKSSINBV (Example 3) is digested with Xho I and Stu I, and the vector is purified by GENECLEAN™ and ligated with the gamma interferon insert. The resulting vector construction is known as pKSSIN $\gamma$ Mu. The human gamma interferon gene (Howard et al., *supra*) is similarly inserted into pKSSINBV using the same strategy. The resulting vector construct is known as pKSSIN $\gamma$ Hu. The interferon expressing Sindbis vectors are then packaged into vector particles. This is accomplished by introducing RNA from these vectors into a packaging cell line as described in Example 7.

The mouse and human interferon genes are also cloned into pVGELVISSINBV-linker (see Example 3). Briefly, pVGELVISSINBV-linker is first digested with Asc I and the termini made blunt by the addition of Klenow enzyme and dNTPs. The Klenow is heat inactivated and the vector is subsequently digested with Xho I. This vector is purified by GENECLEAN™ and ligated to the gamma interferon inserts prepared as described above. The resulting vectors are described pVGELVIS- $\gamma$ Mu and pVGELVIS- $\gamma$ Hu, respectively.

##### 2. INTERLEUKIN-2

The human IL-2 gene is cloned by PCR amplification into the KT-3 retroviral backbone (Howard et al., *Ann. N.Y. Acad. Sci.* 716:167-187, 1994). The source for the IL-2 gene is a pBR322 based plasmid which contains the IL-2 cDNA (ATCC #61391). The cDNA is PCR amplified using a standard three-temperature protocol as described in Example 3. The 5' primer is the sense sequence of the hIL-2 gene complementary to the 5' coding region beginning at the ATG start codon. Additionally, a Xho I site is built into the 5' end of the primer sequence.

5' hIL-2 (SEQ.ID NO. 123)  
5'-GCCTCGAGACAATGTACAGGATGCAACTC-  
CTGTCT

The 3' primer is an antisense sequence of the hIL-2 gene complementary to the 3' coding region ending at the TAA stop codon. Additionally, a Cla I site is built into the 5' end of the primer sequence.

3' hIL-2 (SEQ.ID NO. 124)  
5'-GAATCGATTATCAAGTCAGTGTGGAGAT-  
GATGCT

The PCR amplicon is purified in a 1% agarose gel. To place the IL-2 gene in the KT-3 retroviral backbone, pMu-IFN is digested with Xho I and Cla I to remove the interferon gene. After treatment with phosphatase, the vector is purified in a 1% agarose gel. The vector and IL-2 insert are ligated and

transformed using standard procedures, and recombinant clones are screened by restriction enzyme analysis. The resulting vector is designated pKThIL-2.

Human IL-2 is subcloned from the retroviral vector pKThIL2, into the pKSSINBV vector, using the same strategy employed for murine gamma interferon. The resulting vector construction is known as pKSSIN-huIL-2. The human IL-2 gene is also cloned into pVGELVISSINBV-linker as described above for the gamma interferon genes. The resulting construct is designated pVGELVIS-IL-2.

### 3.HSV-TK

The coding region and transcriptional termination signals of HSV-1 thymidine kinase (HSV-TK) are isolated as a 1.8 kb Bgl II/Pvu II fragment from plasmid 322TK (McKnight et. al., *Nuc. Acids Res.* 8:5949, 1980) cloned into pBR 322 (ATCC No. 31344). The ends are made blunt by the addition of Klenow enzyme and dNTPs. The 1.8 kb fragment is isolated on a 1% agarose gel and ligated to pKSSINBV which had been previously digested with Stu I, phosphatase and gel purified. This construct is known as pKSSINBV-TK. For use is physical gene transfer experiments, the TK gene is similarly cloned into pVGELVIS-SINBV-linker. The vector is prepared by digestion with Pml I, phosphatase treatment and isolated on a 1% agarose gel. This vector construct is known as pVGELVISBV-TK.

### B. ADMINISTRATION

Any of the above-described vector constructs may be utilized along with packaging cell lines described in Example 7, in order to produce recombinant alphavirus particles suitable for administration to humans or animals (either directly or indirectly), or for infecting target cells. Such vector constructs may also introduced directly into target cells as a "naked" DNA molecule, as a DNA complex with various liposome formulations, or as a DNA ligand complex including the alphavirus DNA vector molecule (e.g., along with a polycation compound such as polylysine, a receptor specific ligand, or a psoralen inactivated virus such as Sendai or Adenovirus).

This aspect of the invention relates to pharmaceutical compositions comprising alphavirus vector constructs, recombinant alphavirus particles, or eukaryotic layered vector initiation systems described above (individually and/or collectively referred to herein sometimes as "gene delivery vehicles"), in combination with a pharmaceutically acceptable carrier or diluent. Such gene delivery vehicles can be formulated in crude or, preferably, purified form. Pharmaceutical compositions comprising the gene delivery vehicles may be prepared either as a liquid solution or as a solid form (e.g., lyophilized) which is resuspended in a solution prior to administration. In addition, the composition may be prepared with suitable carriers or diluents for topical administration, injection, or nasal, oral, vaginal, sub-lingual, inhalant, intraocular, enteric, or rectal administration.

Pharmaceutically acceptable carriers or diluents are non-toxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic saline solutions, preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin (HSA).

Gene delivery vehicles according to the invention can be stored in liquid, or preferably, lyophilized form. Factors influencing stability include the formulation (liquid, freeze dried, constituents thereof, etc.) and storage conditions, including temperature, storage container, exposure to light,

etc. Alternatively, pharmaceutical compositions according to the invention can be stored as liquids at low temperatures. In a preferred embodiment, the gene delivery vehicles of the invention are formulated to preserve infectivity in a lyophilized form at elevated temperatures, and for this form to be suitable for injection into patients following reconstitution.

In another aspect of the present invention, methods are provided for preventing or treating various diseases and genetic disorders. Such methods comprise administering a gene delivery vehicle as described above, such that a therapeutically efficacious amount of the desired, or "selected," gene product is produced. As used herein, a "therapeutically effective amount" is an amount that is of clinical relevance, i.e., protective immunity is achieved, tumor progression is retarded, etc. A "therapeutically effective amount" of a gene delivery vehicle according to the invention refers to the amount that must be administered to produce a therapeutically effective amount of the desired gene product in a particular patient or application. For instance, in a patient suffering from hemophilia A, a therapeutically effective amount of a gene delivery vehicle is an amount that elicits production of sufficient factor VIII (the desired gene product expressed from the selected heterologous nucleotide sequence) to produce therapeutically beneficial clotting and will thus generally be determined by each patient's attending physician, although serum levels of about 0.2 ng/mL (about 0.1% of "normal" levels) or more will be therapeutically beneficial. Typical dosages will range from about  $10^5$  to  $10^{12}$  gene delivery vehicles.

In some cases, gene delivery vehicles according to the invention will be administered as an adjunct to other therapy, such as hormonal, radiation, and/or chemotherapeutic treatment.

In various embodiments of the invention, gene delivery vehicles may be administered by various routes in vivo, or ex vivo, as described in greater detail below. Alternatively, the gene delivery vehicles of the present invention may also be administered to a patient by a variety of other methods. Representative examples include transfection by various physical methods, such as lipofection (Felgner, et al., *Proc. Natl. Acad. Sci. USA*, 84:7413, 1989), direct DNA injection (Acsadi, et al., *Nature*, 352:815, 1991; microprojectile bombardment (Williams, et al., *Proc. Nat'l. Acad. Sci. USA*, 88:2726, 1991); liposomes of several types (see e.g., Wang, et al., *Proc. Nat'l. Acad. Sci. USA*, 84:7851, 1987);  $\text{CaPO}_4$  (Dubensky, et al., *Proc. Nat'l. Acad. Sci. USA*, 81:7529, 1984); DNA ligand (Wu, et al., *J. Biol. Chem.*, 264:16985, 1989); or administration of nucleic acids alone (WO 90/11092). Other possible methods of administration can include injection of producer cell lines into the blood or, alternatively, into one or more particular tissues, grafting tissue comprising cells treated with gene delivery vehicles according to the invention, etc.

When pharmaceutical compositions according to the invention are administered in vivo, i.e., to the cells of patient without prior removal of the cells from the patient, administration can be by one or more routes. In this context, "administration" is equivalent to "delivery." Typical routes of administration include traditional parenteral routes, such as intramuscular (i.m.), subcutaneous (sub-q), intravenous (i.v.), and interperitoneal (i.p.) injection. Other suitable routes include nasal, pulmonary, and even direct administration into a particular tissue, such as the liver, bone marrow, etc. In addition, other routes may be employed, as described below.

Transdermal or topical application of a pharmaceutical composition comprising a gene delivery vehicle according

to the invention may be used as an alternate route of administration because the skin is the most expansive and readily accessible organ of the human body. Transdermal delivery systems (TDS) are capable of delivering a gene delivery vehicle through intact skin so that it reaches the systemic circulation in sufficient quantity to be therapeutically effective. TDS provide a variety of advantages, including elimination of gastrointestinal absorption problems and hepatic first pass effect, reduction of dosage and dose intervals, and improved patient compliance. The major components of TDS are a controlled release device composed of polymers, a gene delivery vehicle according to the invention, excipients, and enhancers, and a fastening system to fix the device to the skin. A number of polymers have been described and include, but are not limited to, gelatin, gum arabic, paraffin waxes, and cellulose acetate phthalate (Sogibayasi, et al., *J. Controlled Release*, 29:177, 1994). These polymers can be dermatologically formulated into aqueous, powder, or oil phases. Various combinations can produce lotions, pastes, ointments, creams, and gels, alone or together with the aid of emulsifiers.

Additionally, iontophoresis may be used to cause increased penetration of ionized substances into or through the skin by the application of an electrical field. This method has the advantage of being able to deliver the drug in a pulsatile manner (Singh, et al, *Dermatology*, 187:235, 1993).

Topical administration may also be accomplished by encapsulating gene delivery vehicles according to the invention in liposomes. Hyaluronic acid has been used as a bioadhesive ligand for the formation of liposomes to enhance adherence and retention to the extracellular matrix in cases of burns and wound healing (Yerushalmi, et al., *Arch. Biochem. and Biophys.*, 313:267, 1994). As those in the art will appreciate, methods of liposome preparation can be tailored to control size and morphology. Liposomes can also be made to include one or more targeting elements to target a specific cell type.

Ocular administration is an alternate route to achieve delivery of compositions described herein. Systemic absorption occurs through contact with the conjunctival and nasal mucosae, the latter occurring as the result of drainage through the nasolacrimal duct. Formulations such as those described above which further comprise inert ingredients such as buffers, chelating agents, antioxidants, and preservatives can be incorporated into ophthalmic dosage forms intended for multiple dose use. Formulations also may consist of aqueous suspensions, ointments, gels, inserts, bioadhesives, microparticles, and nanoparticles.

The nasal cavity also offers an alternative route of administration for compositions comprising a gene delivery vehicle as described herein. For instance, the human nasal cavities have a total surface area of approximately 150 cm<sup>2</sup> and are covered by a highly vascular mucosal layer. A respiratory epithelium, comprised of columnar cells, goblet cells, and ciliary cuboidal cells, lines most of the nasal cavity (Chien, et al, *Crit. Rev. in Therap. Drug Car. Sys.*, 4:67, 1987). The subepithelium contains a dense vascular network and the venous blood from the nose passes directly into the systemic circulation, avoiding first-pass metabolism in the liver. Thus, delivery to the upper region of the nasal cavity may result in slower clearance and increased bioavailability of gene delivery vehicles. The absence of cilia in this area is an important factor in the increased effectiveness of nasal sprays as compared to drops. The addition of viscosity-building agents, such as methycellulose, etc. can change the pattern of deposition and clearance of intranasal applications. Additionally, bioadhesives can be used as a means to

prolong residence time in the nasal cavity. Various formulations comprising sprays, drops, and powders, with or without the addition of absorptive enhancers, have been described (see Wearley, L, *supra* ).

Oral administration includes sublingual, buccal, and gastrointestinal delivery. Sublingual and buccal (cheek) delivery allow for rapid systemic absorption of gene delivery vehicles and avoid hepatic first-pass metabolism and degradation in the stomach and intestines. Unidirectional buccal delivery devices can be designed for oral mucosal absorption only. Additionally, these devices can prevent diffusion-limiting mucus buildup to allow for enhanced absorption. Delivery through the gastrointestinal tract allows for precise targeting for drug release. Depending on the formulation, gene delivery vehicles can be specifically delivered to areas in the stomach, duodenum, jejunum, ileum, cecum, colon, or rectum. Oral formulations include tablets, capsules, aqueous suspensions, and gels. These may contain bioadhesive polymers, hydrodynamically balanced systems, gastroinflatable delivery devices, intragastric retention shapes, enteric coatings, excipients, or intestinal absorption promoters (Ritschel, W. A., *Meth. Exp. Clin. Pharmacol.*, 13:313, 1991).

The human rectum has a surface area of between 200 to 400 cm<sup>2</sup> and is abundant in blood and lymphatic vessels. This offers an alternative route for administering compositions according to the invention. Depending on the actual site of administration, it may be possible to bypass first-pass metabolism by the liver. Targeting of the systemic circulation can be achieved by delivering the vehicle to an area behind the internal rectal sphincter which allows absorption directly into the inferior vena cava, thereby bypassing the portal circulation and avoiding metabolism in the liver. The liver can be targeted by delivering the vehicle to the region of the ampulla recti, which allows absorption into the portal system (Ritschel, *supra*).

Alternatively, pulmonary administration can be accomplished through aerosolization. As the lungs are highly vascularized, this type of administration allows systemic delivery. The three systems commonly used for aerosol production are: the nebulizer, the pressurized metered dose inhaler, and the dry powder inhaler, all of which are known in the art. Aerosol therapy is very common in obstructive bronchial diseases but can be used as well as for the treatment of systemic diseases. The surface area of the adult human lung is approximately 75 m<sup>2</sup> and requires only one puff of an aerosol to cover this entire area within seconds. Absorption occurs quickly because the walls of the alveoli in the deep lung are extremely thin. Absorption and clearance depends on a number of factors, including particle size and solubility (Wearley, L, *supra* ). Particles are preferably smaller than 5  $\mu$ M in diameter.

The vaginal mucosa consists of stratified squamous epithelium. Gene delivery vehicles can be administered through the vaginal orifice onto the mucosa. Formulations include ointments, creams, and suppositories. Additional information regarding these and other routes of administration may be found in U.S. Ser. No. 08/366,788.

As an alternative to *in vivo* administration of the gene delivery vehicles of the invention, *ex vivo* administration can be employed. *Ex vivo* treatment envisions withdrawal or removal of a population of cells from a patient. Exemplary cell populations include bone marrow cells, liver cells, and blood cells from the umbilical cord of a newborn. Such cells may be processed to purify desired cells for transduction prior to such procedures, for instance to obtain subsets of such cell populations, e.g., CD34<sup>+</sup> bone marrow progenitor



cells. Preferred methods of purification include various cell sorting techniques, such as antibody panning, FACS, and affinity chromatography using a matrix coupled to antibodies specifically reactive to the desired cell type(s). Isolated cells are then transduced, after which they may be immediately re-introduced to the patient from which they were withdrawn. Alternatively, the cells may be expanded in culture by various techniques known to those skilled in the art prior to re-introduction.

In another embodiment of the invention, gene delivery vehicles of the invention are administered to patients in conjunction with another therapeutic compound. As those in the art will appreciate, such compounds may include, but are not limited to, other gene delivery vehicles designed to deliver one or more other therapeutic genes to the patient, as is described in U.S. Ser. No. 08/368,210.

In accordance with the non-parenteral administration the present invention, the gene delivery vehicles, particularly those comprised of unencapsidated nucleic acid, may be complexed with a polycationic molecule to provide polycation-assisted non-parenteral administration. Such a method of gene delivery facilitates delivery of a gene via mediation by a physical particle comprised of multiple components that augment the efficiency and specificity of the gene transfer. In particular, polycationic molecules, such as polylysine and histone, have been shown to neutralize the negative charges on a nucleic acid molecule and to condense the molecule into a compact form. This form of molecule is transferred with high efficiency in cells, apparently through the endocytic pathway. The uptake in expression of the nucleic acid molecule in the host cell results after a series of steps, as follows: (1) attachment to cell surface; (2) cell entry via endocytosis or other mechanisms; (3) cytoplasmic compartment entry following endosome release; (4) nuclear transport; and (5) expression of the nucleic acid molecule carried by the gene delivery vehicle. In a further preferred embodiment, multi-layer technologies are applied to the polycation-nucleic acid molecule complex to facilitate completion of one or more of these steps. For example, a ligand such as asialoglycoprotein, transferrin, and immunoglobulin may be added to the complex to facilitate binding of the cell complex to the cell surface, an endosomal disruption component (e.g., a viral protein, a fusogenic peptide such as the n-terminus of the influenza virus hemagglutinin or an inactivated virus) is added to facilitate the release of DNA from the endosome, or a nuclear protein (or a peptide containing a nuclear localization signal) is added to facilitate the transport of the DNA into the nucleus. In a further preferred embodiment, the composition comprising the complex includes inactivated adenovirus particles (Curiel, D.T., et al., *PNAS* 88: 8850-8854, 1991; Cristiano, R.J., *PNAS* 90: 2122-2126 1993; Cotten, M., et al., *PNAS* 89: 6094-6098 1992; Lozier, J.N., et al., *Human Gene Therapy* 5: 313-322, 1994; Curiel, D.T., et al., *Human Gene Therapy* 3: 147-154, 1992; Plank, C. et al., *Bioconjugate Chem.* 3: 533-539, 1992; Wagner, E. et al., *PNAS* 88: 4255-4259, 1991). The assorted components comprising the multi-layer complex may be varied as desired, so that the specificity of the complex for a given tissue, or the gene expressed from the gene delivery vehicle, may be varied to better suit a particular disease or condition.

As noted above, various methods may be utilized to administer gene delivery vehicles of the present invention, including nucleic acids which encode the immunogenic portion(s) discussed above, to warm-blooded animals such as humans, directly. Suitable methods include, for example, various physical methods such as direct DNA injection

(Acsadi et al., *Nature* 352:815-818, 1991), and microprojectile bombardment (Williams et al., *PNAS* 88:2726-2730, 1991).

Within an in vivo context, the gene delivery vehicle can be injected into the interstitial space of tissues including muscle, brain, liver, skin, spleen or blood (see, WO 90/11092). Administration may also be accomplished by intravenous injection or direct catheter infusion into the cavities of the body (see, WO 93/00051), discussed in more detail below.

It is generally preferred that administration of the gene delivery vehicles at multiple sites be via at least two injections. In this regard, suitable modes of administration include intramuscular, intradermal and subcutaneous injections, with at least one of the injections preferably being intramuscular. In particularly preferred embodiments, two or more of the injections are intramuscular. However, although administration via injections is preferred, it will be evident that the gene delivery vehicles may be administered through multiple topical or separate ocular administrations. Further, a number of additional routes are suitable for use within the present invention when combined with one or more of the routes briefly noted above, including intraperitoneal, intracranial, oral, rectal, nasal, vaginal and sublingual administration. Methods of formulating and administering the gene delivery vehicles at multiple sites through such routes would be evident to those skilled in the art and are described in U.S. Ser. No. 08/366,788 and U.S. Ser. No. 08/367,071 incorporated herein by reference in their entirety.

#### C. Liposome Formulation

Several methods may be used in the preparation of liposomes to incorporate gene delivery vehicles of the invention, particularly those that are DNA or RNA, see Gregoriadis et al., (*Liposome Technology*, CFC Press, New York 1984), Ostro et al., (*Liposomes*, Marek Dekker, 1987) and Lichtenberg et al., (*Meth. Biochem. Anal.* 33:337, 1988). According to one embodiment of the invention, the gene delivery vehicles are complexed with cationic liposomes or lipid vesicles. Cationic liposome formulations may be prepared from a mixture of positively charged lipids, negatively charged lipids, neutral lipids and cholesterol or similar sterol. The positively charged lipids may be DMRIE (Felgner, et al., *J. Biol. Chem.* 269:1, 1994), DOTMA, DOTAP or analogs thereof or a combination of two or more of these lipids. DMRIE is described in U.S. Ser. No. 07/686,746 which is hereby incorporated reference. The neutral and negatively charged lipids can be any natural or synthetic phospholipid or mono-, di- or triglycerols. The natural phospholipids may be derived from animal and plant sources. For example, natural phospholipids such as phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidylserine, or phosphatidylinositol may be utilized. Synthetic phospholipids may be selected from those having fatty acid groups such as dimyristoylphosphatidylcholine, distearoylphosphatidylcholine, dipalmitoylphosphatidylcholine, distearoylphosphatidylcholine, and the corresponding phosphatidylethanolamines and phosphatidylglycerols. The neutral lipids may be phosphatidylcholine, cardiolipin, phosphatidylethanolamine, mono-, di- or triacylglycerols, or analogs thereof such as dioleoylphosphatidylethanolamine (DOPE). The negatively charged lipids may be phosphatidylglycerol, phosphatidic acid or a similar phospholipid analog. Other additive known to those skilled in the art may also be used such as cholesterol, glycolipids, fatty

acids, sphingolipids, prostaglandins, gangliosides, neobee, niomes, or any other natural or synthetic amphophiles.

Substitution of the cationic lipid component of liposomes may be used to alter the transfection efficiency of the liposome. For example, 1,2-dimyristyloxypropyl-3-  
dimethyl-hydroxyethyl ammonium bromide (DMRIE) is  
used in conjunction with DOPE which provides increased  
transfection efficiency and does not aggregate at high con-  
centrations as other formulations such as DC-cholesterol/  
DOPE. These characteristics allows for higher absolute  
concentrations of DNA and liposomes to be introduced into  
patients in vivo without increased levels of toxicity. A  
preferred molar ratio of DMRIE to DOPE of 9:1 to 1:9 with  
a particularly preferred molar ratio of 5:5 (see WO 94/29469  
incorporated herein by reference)

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Additionally, the publications and other materials cited to illuminate the background of the invention, and in particular, to provide additional details concerning its practice as described in the detailed description and examples, are hereby incorporated by reference in their entirety.

A Sequence Listing has also been included herewith in accordance with the provisions of 37 C.F.R. § 1.821 et seq. To the extent any discrepancy exists between the Specification Figures and the Sequence Listing, the specification or Figures should be considered to be the primary document.

## SEQUENCE LISTING

## ( 1 ) GENERAL INFORMATION:

( 1 1 1 ) NUMBER OF SEQUENCES: 128

## ( 2 ) INFORMATION FOR SEQ ID NO:1:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 16656 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATTGACGGCG	TAGTACACAC	TATTGAATCA	AACAGCCGAC	CAATCGCACT	ACCATCACAA	60
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CGACGATCTT	GGACATAGGC	AGCGCACCCG	CTCGTAGAAT	GTTTTCCGAG	CACCAGTATC	300
ATTGTGTCTG	CCCCATGCGT	AGTCCAGAAG	ACCCGGACCG	CATGATGAAA	TATGCCAGTA	360
AACTGGCGGA	AAAAGCGTGC	AAGATTACAA	ACAAGAAGTT	GCATGAGAAG	ATTAAGGATC	420
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TTACCTGCAA	CATGCGTGCC	GAATATTCCG	TCATGCAGGA	CGTGTATATC	AACGCTCCCG	540
GAACATATCTA	TCATCAGGCT	ATGAAAAGCG	TGCGGACCCT	GTAATGGATT	GGCTTCGACA	600
CCACCCAGTT	CATGTTCTCG	GCTATGGCAG	GTTCGTACCC	TGCGTACAAC	ACCAACTGGG	660
CCGACGAGAA	AGTCCTTGAA	GCGCGTAACA	TCGGACTTTG	CAGCACAAAG	CTGAGTGAAG	720
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TTCCATCGGT	GTTCCACTTG	AATGGAAAGC	AGTCGTACAC	TTGCCGCTGT	GATACAGTGG	900
TGAGTTGCGA	AGGCTACGTA	GTGAAGAAAA	TCACCATCAG	TCCCGGGATC	ACGGGAGAAA	960
CCGTGGGATA	CGCGGTTACA	CACAATAGCG	AGGGCTTCTT	GCTATGCAAA	GTTACTGACA	1020
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TTGGGCTCAA	CCAGCGAATT	GTCATTAACG	GTAGGACTAA	CAGGAACACC	AACACCATGC	1200
AAAATTACCT	TCTGCCGATC	ATAGCACAAAG	GGTTTCAGCAA	ATGGGCTAAG	GAGCGCAAGG	1260

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CAGCGGCAAG	AAAAGAGCCC	ACTCCACCGG	CAAGCAATAG	CTCTGAGTCC	CTCCACCTCT	5460
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CAGCGGTACA	ACCCCTGGCA	ACAGGCCCCA	CGGATGTGCC	TATGTCTTTC	GGATCGTTTT	5580
CCGACGGAGA	GATTGATGAG	CTGAGCCGCA	GAGCAACTGA	GTCCGAACCC	GTCCTGTTTG	5640
GATCATTTGA	ACCGGGCGAA	GTGAACTCAA	TTATATCGTC	CCGATCAGCC	GTATCTTTTC	5700
CACTACGCAA	GCAGAGACGT	AGACGCAGGA	GCAGGAGGAC	TGAATACTGA	CTAACCGGGG	5760
TAGGTGGGTA	CATATTTTTCG	ACGGACACAG	GCCCTGGGCA	CTTGCAAAAAG	AAGTCCGTTT	5820
TGCAGAACCA	GCTTACAGAA	CCGACCTTGG	AGCGCAATGT	CCTGGAAAAGA	ATTCATGCCC	5880
CGGTGCTCGA	CACGTCGAAA	GAGGAACAAC	TCAAACTCAG	GTACCAGATG	ATGCCACCGG	5940
AAGCCAACAA	AAGTAGGTAC	CAGTCTCGTA	AAGTAGAAAA	TCAGAAAGCC	ATAACCACTG	6000
AGCGACTACT	GTCAGGACTA	CGACTGTATA	ACTCTGCCAC	AGATCAGCCA	GAATGCTATA	6060

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AGATCACCTA	TCCGAAACCA	TTGTACTCCA	GTAGCGTACC	GGCGAACTAC	TCCGATCCAC	6120
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ATCAGATTAC	TGACGAGTAC	GATGCTTACT	TGGATATGGT	AGACGGGACA	GTCGCCTGCC	6240
TGGATACTGC	AACCTTCTGC	CCCCTAAGC	TTAGAAAGTTA	CCCGAAAAAA	CATGAGTATA	6300
GAGCCCCGAA	TATCCGCAGT	GCGGTTCCAT	CAGCGATGCA	GAACACGCTA	CAAAATGTGC	6360
TCATTGCCGC	AACTAAAAGA	AATTGCAACG	TCACGCAGAT	GCGTGAACTG	CCAACACTGG	6420
ACTCAGCGAC	ATTCAATGTC	GAATGCTTTT	GAAAAATATGC	ATGTAATGAC	GAGTATTGGG	6480
AGGAGTTTCG	TCGGAAGCCA	ATTAGGATTA	CCACTGAGTT	TGTCACCGCA	TATGTAGCTA	6540
GACTGAAAGG	CCCTAAGGCC	GCCACACTAT	TTGCAAAGAC	GTATAATTTG	GTCCCATTGC	6600
AAGAAGTGCC	TATGGATAGA	TTCGTCATGG	ACATGAAAAAG	AGACGTGAAA	GTTACACCAG	6660
GCACGAAACA	CACAGAAGAA	AGACCGAAAG	TACAAAGTGAT	ACAAGCCGCA	GAACCCCTGG	6720
CGACTGCTTA	CTTATGCGGG	ATTCACCGGG	AATTAAGTGC	TAGGCTTACG	GCCGTCTTGC	6780
TTCCAAACAT	TCACACGCTT	TTTGACATGT	CGGCGGAGGA	TTTTGATGCA	ATCATAGCAG	6840
AACACTTCAA	GCAAGGCGAC	CCGGTACTGG	AGACGGATAT	CGCATCATTC	GACAAAAGCC	6900
AAGACGACGC	TATGGCGTTA	ACCGGTCTGA	TGATCTTGGA	GGACCTGGGT	GTGGATCAAC	6960
CACTACTCGA	CTTGATCGAG	TGCGCCTTTG	GAGAAATATC	ATCCACCCAT	CTACCTACGG	7020
GTA CTGTTT	TAAATTGCGG	GCGATGATGA	AATCCGGAAT	GTTCTCACA	CTTTTTGTCA	7080
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GCCCCTTCCC	GGCCCCCACT	GCCATGTGGA	GGCCGCGGAG	AAGGAGGCAG	GCGGCCCCGA	7740
TGCCTGCCCC	CAACGGGCTG	GCTTCTCAAA	TCCAGCAACT	GACCACAGCC	GTCAGTGCCC	7800
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CAAAGCTCAA	ATTTACCAAG	TCGTACAGCAT	ACGACATGGA	GTTTCGCACAG	TTGCCAGTCA	8160
ACATGAGAAAG	TGAGGCATTC	ACCTACACCA	GTGAACACCC	CGAAGGATTC	TATAACTGGC	8220
ACCACGGAGC	GGTGACGTAT	AGTGGAGGTA	GATTTACCAT	CCCTCGCGGA	GTAGGAGGCA	8280
GAGGAGACAG	CGGTGCTCCG	ATCATGGATA	ACTCCGGTCG	GGTTGTCGCG	ATAGTCCTCG	8340
GTGGCGCTGA	TGAAGGAACA	CGAACTGCCC	TTTCGGTCTG	CACCTGGAAT	AGTAAAGGGA	8400
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AATGCCCTCC	AGGGGACAGC	GTAACGGTTA	GCATAGTGAG	TAGCAACTCA	GCAACGTCAT	9000
GTACACTGGC	CCGCAAGATA	AAACCAAAAT	TCGTGGGACG	GGAAAAATAT	GATCTACCTC	9060
CCGTTACAGG	TAAAAGAATT	CCTTGACAG	TGTACGACCG	TCTGAAAACA	ACTGCAGGCT	9120
ACATCACTAT	GCACAGGCCG	GGACCGCACG	CTTATACATC	CTACCTGGAA	GAATCATCAG	9180
GGAAAGTTTA	CGCAAAGCCG	CCATCTGGGA	AGAACATTAC	GTATGAGTGC	AAGTGCGGCG	9240
ACTACAAGAC	CGGAACCGTT	TCGACCCGCA	CCGAAATCAC	TGGTTGCACC	GCCATCAAGC	9300
AGTGCGTCGC	CTATAAGAGC	GACCAAACGA	AGTGGGTCTT	CAACTCACCG	GACTTGATCA	9360
GACATGACGA	CCACACGGCC	CAAGGGAAAT	TGCATTTGCC	TTTCAAGTTG	ATCCCGGGTG	9420
CCTGCATGGT	CCCTGTTGCC	CACGCGCCGA	ATGTAATACA	TGGCTTTAAA	CACATCAGCC	9480
TCCAATTAGA	TACAGACCAC	TTGACATTGC	TCACCACCAG	GAGACTAGGG	GCAAACCCGG	9540
AACCAACCAC	TGAATGGATC	GTCGGAAAGA	CGGTCAGAAA	CTTCACCGTC	GACCGAGATG	9600
GCCTGGAATA	CATATGGGGA	AATCATGAGC	CAGTGAGGGT	CTATGCCCAA	GAGTCAGCAC	9660
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CAGTGTTATG	TGCCTGTAAA	GCGCGCCGTG	AGTGCCTGAC	GCCATACGCC	CTGGCCCCAA	9840
ACGCCGTAAAT	CCCAACTTCG	CTGGCACTCT	TGTGCTGCGT	TAGGTCGGCC	AATGCTGAAA	9900
CGTTACCCGA	GACCATGAGT	TACTTGTGGT	CGAACAGTCA	GCCGTTCTTC	TGGGTCCAGT	9960
TGTGCATACC	TTTGGCCGCG	TTCATCGTTC	TAATGCGCTA	CTGCTCCTGC	TGCCTGCCTT	10020
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TCAATTTGGA	GATCACTGTC	ATGTCCTCGG	AGGTTTTGCC	TTCCACCAAC	CAAGAGTACA	10200
TTACCTGCAA	ATTCACTACT	GTGGTCCCCT	CCCCAAAAAT	CAAATGCTGC	GGCTCCTTGG	10260
AATGTCAGCC	GGCCGCTCAT	GCAGACTATA	CCTGCAAGGT	CTTCGGAGGG	GTCTACCCCT	10320
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CCGCGATGAA	AGTAGGACTG	CGTATAGTGT	ACGGGAACAC	TACCA GTTTC	CTAGATGTGT	10500
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CAGCATCGTT	TACGCCATTG	GATCATAAGG	TCGTTATCCA	TCGCGGCCTG	GTGTACAACT	10620
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CAGGCCGCCC	ACTGCAGGAA	ACCGCACCTT	TCGGGTGTAA	GATTGCAGTA	AATCCGCTCC	10860

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CCTCCCCCGT	GCCTTCCTTG	ACCCTGGAAG	GTGCCACTCC	CACTGTCCTT	TCCTAATAAA	11880
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GCTCCCCAOC	AGGCAGAAAT	ATGCAAAGCA	TGCATCTCAA	TTAGTCAGCA	ACCATAGTCC	12660
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TCCAGAAGTA	GTGAGGAGGC	TTTTTTGGAG	GCCTAGGCTT	TTGCAAAAAG	CTCCCGGGAG	12840
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GGCGCCCGGT	TCTTTTTGTC	AAGACCGACC	TGTCCGGTGC	CCTGAATGAA	CTGCAGGACG	13080
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CTTATTTGAA	CTAACCAATC	AGTTTCGTTT	TCGCTTCTGT	TCGCGCGCTT	CTGCTCCCCG	16620
AGCTCAATAA	AAGAGCCAC	AACCCCTCAC	TCGGGG			16656

## ( 2 ) INFORMATION FOR SEQ ID NO:2:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 24 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATCTCTACGG	TGGTCCTAAA	TAGT	24
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## ( 2 ) INFORMATION FOR SEQ ID NO:3:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 42 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TATATTCTAG	ATTTTTTTTT	TTTTTTTTTT	TTTTTTGAAA	TG	42
------------	------------	------------	------------	----	----

## ( 2 ) INFORMATION FOR SEQ ID NO:4:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 48 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TATATGGGCC	CGATTTAGGT	GACACTATAG	ATTGACGGCG	TAGTACAC	48
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## ( 2 ) INFORMATION FOR SEQ ID NO:5:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 23 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single

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( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTGGCAACCG GTAAGTACGA TAC 2 3

( 2 ) INFORMATION FOR SEQ ID NO:6:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 21 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATACTAGCCA CGGCCGGTAT C 2 1

( 2 ) INFORMATION FOR SEQ ID NO:7:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 21 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCCTCTTTCG ACGTGTCGAG C 2 1

( 2 ) INFORMATION FOR SEQ ID NO:8:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 21 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACCTTGGAGC GCAATGTCCT G 2 1

( 2 ) INFORMATION FOR SEQ ID NO:9:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 21 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCTTTTCAGG GGATCCGCCA C 2 1

( 2 ) INFORMATION FOR SEQ ID NO:10:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 21 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTGGCGGATC CCCTGAAAAG G 2 1

( 2 ) INFORMATION FOR SEQ ID NO:11:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

-continued

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( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGGGCGCGTGT GGTCTCATG 2 0

( 2 ) INFORMATION FOR SEQ ID NO:12:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 21 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGGGTCTTCA ACTCACCGGA C 2 1

( 2 ) INFORMATION FOR SEQ ID NO:13:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 22 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CAATTGACG TACGCCTCAC TC 2 2

( 2 ) INFORMATION FOR SEQ ID NO:14:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 22 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GAGTGAGGCG TACGTCGAAT TG 2 2

( 2 ) INFORMATION FOR SEQ ID NO:15:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 33 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TATATAGATC TAATGAAAGA CCCCACCTGT AGG 3 3

( 2 ) INFORMATION FOR SEQ ID NO:16:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 40 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TCAATCCCCG AGTGAGGGGT TGTGGGCTCT TTTATTGAGC 4 0

( 2 ) INFORMATION FOR SEQ ID NO:17:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 36 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:17:

-continued

CCACAACCCC TCACTCGGGG ATTGACGGCG TAGTAC

3 6

## ( 2 ) INFORMATION FOR SEQ ID NO:18:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 23 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTGGCAACCG GTAAGTACGA TAC

2 3

## ( 2 ) INFORMATION FOR SEQ ID NO:19:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 22 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGTAACAAGA TCTCGTGCCG TG

2 2

## ( 2 ) INFORMATION FOR SEQ ID NO:20:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 53 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TATATATATA TGCGGCCGCT TTCTTTTATT AATCAACAAA ATTTTGTTTT TAA

5 3

## ( 2 ) INFORMATION FOR SEQ ID NO:21:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 48 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TATATGAGCT CTTTTTTTTT TTTTTTTTTT TTTTTTGAAA TGTAAAAA

4 8

## ( 2 ) INFORMATION FOR SEQ ID NO:22:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TATATCTCGA GGGTGGTGTT GTAGTATTAG TCAG

3 4

## ( 2 ) INFORMATION FOR SEQ ID NO:23:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 43 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TATATGGGCC CTTAAGACCA TCGGAGCGAT GCTTTATTTT CCC

4 3

-continued

## ( 2 ) INFORMATION FOR SEQ ID NO:24:

- ( i ) SEQUENCE CHARACTERISTICS:
- ( A ) LENGTH: 18 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:24:

T C T C T A C G G T G G T C C T A A

1 8

## ( 2 ) INFORMATION FOR SEQ ID NO:25:

- ( i ) SEQUENCE CHARACTERISTICS:
- ( A ) LENGTH: 5 amino acids
  - ( B ) TYPE: amino acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:25:

S e r L e u A r g T r p S e r  
1 5

## ( 2 ) INFORMATION FOR SEQ ID NO:26:

- ( i ) SEQUENCE CHARACTERISTICS:
- ( A ) LENGTH: 26 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:26:

C A T C T C T A C G G T G G T C C T A A A T A G T C

2 6

## ( 2 ) INFORMATION FOR SEQ ID NO:27:

- ( i ) SEQUENCE CHARACTERISTICS:
- ( A ) LENGTH: 34 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:27:

T C G A G A C T A T T T A G G A C C A C C G T A G A G A T G G G C C

3 4

## ( 2 ) INFORMATION FOR SEQ ID NO:28:

- ( i ) SEQUENCE CHARACTERISTICS:
- ( A ) LENGTH: 25 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:28:

C C C T T G T A C G G C T A A C C T A A A G G A C

2 5

## ( 2 ) INFORMATION FOR SEQ ID NO:29:

- ( i ) SEQUENCE CHARACTERISTICS:
- ( A ) LENGTH: 33 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:29:

T C G A G T C C T T T A G G T T A G C C G T A C A A G G G G G C C

3 3

-continued

## ( 2 ) INFORMATION FOR SEQ ID NO:30:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 26 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CATCGCTACG GTGGTCCTAA ATAGTC

2 6

## ( 2 ) INFORMATION FOR SEQ ID NO:31:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 34 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TCGAGACTAT TTAGGACCAC CGTAGCGATG GGCC

3 4

## ( 2 ) INFORMATION FOR SEQ ID NO:32:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 48 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CGGAAATAAA GCATCTCTAC GGTGGTCCTA AATAGTCAGC ATAGTACC

4 8

## ( 2 ) INFORMATION FOR SEQ ID NO:33:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 56 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TCGAGGTACT ATGCTGACTA TTTAGGACCA CCGTAGAGAT GCTTTATTTT CCGGGCC

5 6

## ( 2 ) INFORMATION FOR SEQ ID NO:34:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 41 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TATATGCGGC CGCTCTAGAT TACAATTGG ACTTTCCGCC C

4 1

## ( 2 ) INFORMATION FOR SEQ ID NO:35:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 44 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:35:

TATATATGAG CTCTTACAAA TAAAGCAATA GCATCACAAA TTTC

4 4

## ( 2 ) INFORMATION FOR SEQ ID NO:36:

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( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 36 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:36:

TATATGAATT CGTTTGGACA AACCACAACT AGAATG 3 6

( 2 ) INFORMATION FOR SEQ ID NO:37:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 44 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TATATATGAG CTCTAATAAA ATGAGGAAAT TGCATCGCAT TGTC 4 4

( 2 ) INFORMATION FOR SEQ ID NO:38:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 43 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TATATGAATT CATAGAATGA CACCTACTCA GACAATGCGA TGC 4 3

( 2 ) INFORMATION FOR SEQ ID NO:39:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 46 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:39:

TATATGAGCT CGGGTCGGCA TGGCATCTCC ACCTCCTCGC GGTCCG 4 6

( 2 ) INFORMATION FOR SEQ ID NO:40:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 52 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:40:

TCCACCTCCT CGCGGTCCGA CCTGGGCATC CGAAGGAGGA CGCACGTCCA CT 5 2

( 2 ) INFORMATION FOR SEQ ID NO:41:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 48 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TATATGAGCT CCTCCCTTAG CCATCCGAGT GGACGTGCGT CCTCCTTC 4 8

( 2 ) INFORMATION FOR SEQ ID NO:42:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 47 base pairs

-continued

( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TATATGCGGC CGCTTTCTTT TATTAATCAA CAAAATTTTG TTTTAA

47

( 2 ) INFORMATION FOR SEQ ID NO:43:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 37 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:43:

TATATGAGCT CGAAATGTTA AAAACAAAAT TTTGTTG

37

( 2 ) INFORMATION FOR SEQ ID NO:44:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 34 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:44:

TATATATAGA TCTTTGACAT TGATTATTGA CTAG

34

( 2 ) INFORMATION FOR SEQ ID NO:45:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 42 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:45:

CCGTCAATAC GGTTCACTAA ACGAGCTCTG CTTATATAGA CC

42

( 2 ) INFORMATION FOR SEQ ID NO:46:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 38 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:46:

GCTCGTTTAG TGAACCGTAT TGACGGCGTA GTACACAC

38

( 2 ) INFORMATION FOR SEQ ID NO:47:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 33 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:47:

TATATATAGA TCTGGTGTGG AAAGTCCCCA GGC

33

( 2 ) INFORMATION FOR SEQ ID NO:48:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 31 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single



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( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CTACGCCGTC AATGCCGAGG CGGCCTCGGC C 3 1

( 2 ) INFORMATION FOR SEQ ID NO:49:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 37 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:49:

GGCCGCCTCG GCATTGACGG CGTAGTACAC ACTATTG 3 7

( 2 ) INFORMATION FOR SEQ ID NO:50:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 41 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:50:

TATATATCTC GAGAAGCTCT AAGGTAAATA TAAAAATTAC C 4 1

( 2 ) INFORMATION FOR SEQ ID NO:51:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 38 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:51:

TATATATCTC GAGAGGTTGG AATCTAAAT ACACAAAC 3 8

( 2 ) INFORMATION FOR SEQ ID NO:52:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 43 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:52:

TATATATGCG GCCGCAAGCT CTAAGGTAA TAAAAATTT ACC 4 3

( 2 ) INFORMATION FOR SEQ ID NO:53:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 40 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:53:

TATATATGCG GCCGCAGGTT GGAATCTAA ATACACAAAC 4 0

( 2 ) INFORMATION FOR SEQ ID NO:54:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 35 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

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( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:54:

TCGAGCACGT GCGCGCCTG ATCACGCGTA GGCCT 3 5

( 2 ) INFORMATION FOR SEQ ID NO:55:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 35 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CTAGAGGCCT ACGCGTGATC AGGCGCGCCA CGTGC 3 5

( 2 ) INFORMATION FOR SEQ ID NO:56:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 35 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:56:

TATATCTCCA GATGAGGTAC ATGATTTTAG GCTTG 3 5

( 2 ) INFORMATION FOR SEQ ID NO:57:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 40 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:57:

TATATATCGA TTCAAGGCAT TTTCTTTTCA TCAATAAAAC 4 0

( 2 ) INFORMATION FOR SEQ ID NO:58:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 35 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:58:

TATATCTCCA GATGATGACA ATGTGGTGTC TGACG 3 5

( 2 ) INFORMATION FOR SEQ ID NO:59:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 32 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:59:

TATATATCGA TTCATGACGA CCGACCTTG CG 3 2

( 2 ) INFORMATION FOR SEQ ID NO:60:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 28 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:60:

-continued

TATATGGGCC CCCCCCCCCC CCCCACG

2 8

( 2 ) INFORMATION FOR SEQ ID NO:61:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 30 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:61:

TATATATCGA TCCCCCCCCC CCCCCCAACG

3 0

( 2 ) INFORMATION FOR SEQ ID NO:62:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 34 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:62:

TATATCCATG GCTTACAATC GTGGTTTTCA AAGG

3 4

( 2 ) INFORMATION FOR SEQ ID NO:63:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 33 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:63:

TATATGGGCC CTCGATGAGT CTGGACGTTT CTC

3 3

( 2 ) INFORMATION FOR SEQ ID NO:64:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 33 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:64:

TATATATCGA TTCGATGAGT CTGGACGTTT CTC

3 3

( 2 ) INFORMATION FOR SEQ ID NO:65:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 37 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:65:

TATATCCATG GATCCAATTT GCTTTATGAT AACAATC

3 7

( 2 ) INFORMATION FOR SEQ ID NO:66:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 30 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:66:

TATATGGGCC CGGTCGACGC CGGCCAAGAC

3 0

-continued

## ( 2 ) INFORMATION FOR SEQ ID NO:67:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 30 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:67:

TATATATCGA TGGTCGACGC CGGCCAAGAC 3 0

## ( 2 ) INFORMATION FOR SEQ ID NO:68:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 32 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:68:

TATATCCATG GTGCCAGCCA GTTGGGCAGC AG 3 2

## ( 2 ) INFORMATION FOR SEQ ID NO:69:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 23 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:69:

TTAATTAACG GCCGCCACCA TGG 2 3

## ( 2 ) INFORMATION FOR SEQ ID NO:70:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 13 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:70:

TAACGGCCGC CAC 1 3

## ( 2 ) INFORMATION FOR SEQ ID NO:71:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 20 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:71:

CCATGGTGGC GGCCGTTAAT 2 0

## ( 2 ) INFORMATION FOR SEQ ID NO:72:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 16 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:72:

GGTTTAAACA GGAGCT 1 6

## ( 2 ) INFORMATION FOR SEQ ID NO:73:

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( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 16 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:73:

CCTGTTTAAA CCAGCT 1 6

( 2 ) INFORMATION FOR SEQ ID NO:74:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 47 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:74:

TATATGCGGC CGCACCACCA CCATGAATAG AGGATTCTTT AACATGC 4 7

( 2 ) INFORMATION FOR SEQ ID NO:75:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 34 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:75:

TATATGCGGC CGCTCATCTT CGTGTGCTAG TCAG 3 4

( 2 ) INFORMATION FOR SEQ ID NO:76:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 61 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:76:

TATATGCGGC CGCATCTCTA CGGTGGTCCT AAATAGTACC ACCACCATGA ATAGAGGATT 6 0  
 C 6 1

( 2 ) INFORMATION FOR SEQ ID NO:77:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 25 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:77:

CTCATCGATC AGATCTGACT AGTTG 2 5

( 2 ) INFORMATION FOR SEQ ID NO:78:

( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 33 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:78:

GATCCAACCTA GTCAGATCTG ATCGATGAGG GCC 3 3

( 2 ) INFORMATION FOR SEQ ID NO:79:

-continued

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 56 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:79:

ACTTATCGAT GGTTC TAGAC TCCCTTAGCC ATCCGAGTGG ACGTGCGTCC TCCTTC 5 6

## ( 2 ) INFORMATION FOR SEQ ID NO:80:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 52 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:80:

TCCACCTCCT CGCGGTCCGA CCTGGGCATC CGAAGGAGGA CGCACGTCCA CT 5 2

## ( 2 ) INFORMATION FOR SEQ ID NO:81:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 57 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:81:

TCCGACCGCG AGGAGGTGGA GATGCCATGC CGACCCATTG ACGGCGTAGT ACACACT 5 7

## ( 2 ) INFORMATION FOR SEQ ID NO:82:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 36 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:82:

CTGGACTAGT TAATACTGGT GCTCGGAAAA CATTCT 3 6

## ( 2 ) INFORMATION FOR SEQ ID NO:83:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 40 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:83:

GTCAAGCTTG CTAGCTACAA CACCACCACC ATGAATAGAG 4 0

## ( 2 ) INFORMATION FOR SEQ ID NO:84:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 40 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:84:

CAGTCTCGAG TTA CTACCAC TCTTCTGTCC CTTCCGGGGT 4 0

## ( 2 ) INFORMATION FOR SEQ ID NO:85:

## ( i ) SEQUENCE CHARACTERISTICS:

-continued

- ( A ) LENGTH: 43 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:85:

TATATGCGGC CGCACCACCA TGTCCGCAGC ACCACTGGTC ACG

4 3

( 2 ) INFORMATION FOR SEQ ID NO:86:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:86:

TATATAGATC TCTTGATCAG CTTCAGAAGA TGGC

3 4

( 2 ) INFORMATION FOR SEQ ID NO:87:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 24 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:87:

TCAATGGCGG GAAGAGGCGG TTGG

2 4

( 2 ) INFORMATION FOR SEQ ID NO:88:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 31 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:88:

CGGCCTCTTC CCGCCATTGA CGGCGTAGTA C

3 1

( 2 ) INFORMATION FOR SEQ ID NO:89:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:89:

TATATAGATC TCTTGATCAG CTTCAGAAGA TGGC

3 4

( 2 ) INFORMATION FOR SEQ ID NO:90:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 44 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:90:

TATATATATG CGGCCGCACC GCCAAGATGT TCCCGTTCCA GCCA

4 4

( 2 ) INFORMATION FOR SEQ ID NO:91:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 38 base pairs
- ( B ) TYPE: nucleic acid

-continued

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( C ) STRANDEDNESS: single	
( D ) TOPOLOGY: linear	
( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:91:	
TATATATATG CGGCCGCTCA ATTATGTTTC TGGTTGGT	3 8
( 2 ) INFORMATION FOR SEQ ID NO:92:	
( i ) SEQUENCE CHARACTERISTICS:	
( A ) LENGTH: 35 base pairs	
( B ) TYPE: nucleic acid	
( C ) STRANDEDNESS: single	
( D ) TOPOLOGY: linear	
( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:92:	
CTCGAGCTCG AGGCACCAGC ACCATGCAAC TTTTT	3 5
( 2 ) INFORMATION FOR SEQ ID NO:93:	
( i ) SEQUENCE CHARACTERISTICS:	
( A ) LENGTH: 29 base pairs	
( B ) TYPE: nucleic acid	
( C ) STRANDEDNESS: single	
( D ) TOPOLOGY: linear	
( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:93:	
CTACTAGATC CCTAGATGCT GGATCTTCC	2 9
( 2 ) INFORMATION FOR SEQ ID NO:94:	
( i ) SEQUENCE CHARACTERISTICS:	
( A ) LENGTH: 29 base pairs	
( B ) TYPE: nucleic acid	
( C ) STRANDEDNESS: single	
( D ) TOPOLOGY: linear	
( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:94:	
GGAAGATCCA GCATCTAGGG ATCTAGTAG	2 9
( 2 ) INFORMATION FOR SEQ ID NO:95:	
( i ) SEQUENCE CHARACTERISTICS:	
( A ) LENGTH: 26 base pairs	
( B ) TYPE: nucleic acid	
( C ) STRANDEDNESS: single	
( D ) TOPOLOGY: linear	
( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:95:	
GGGCGATATC AAGCTTATCG ATACCG	2 6
( 2 ) INFORMATION FOR SEQ ID NO:96:	
( i ) SEQUENCE CHARACTERISTICS:	
( A ) LENGTH: 26 base pairs	
( B ) TYPE: nucleic acid	
( C ) STRANDEDNESS: single	
( D ) TOPOLOGY: linear	
( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:96:	
GGGCGATATC AAGCTTATCG ATACCG	2 6
( 2 ) INFORMATION FOR SEQ ID NO:97:	
( i ) SEQUENCE CHARACTERISTICS:	
( A ) LENGTH: 19 base pairs	
( B ) TYPE: nucleic acid	
( C ) STRANDEDNESS: single	
( D ) TOPOLOGY: linear	



( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:97:

AATACGACTC ACTATAGGG

19

( 2 ) INFORMATION FOR SEQ ID NO:98:

- ( i ) SEQUENCE CHARACTERISTICS:
- ( A ) LENGTH: 29 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:98:

CTACTAGATC CCTAGATGCT GGATCTTCC

29

( 2 ) INFORMATION FOR SEQ ID NO:99:

- ( i ) SEQUENCE CHARACTERISTICS:
- ( A ) LENGTH: 17 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:99:

ATTAACCCTC ACTAAAG

17

( 2 ) INFORMATION FOR SEQ ID NO:100:

- ( i ) SEQUENCE CHARACTERISTICS:
- ( A ) LENGTH: 29 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:100:

GGAAGATCCA GCATCTAGGG ATCTAGTAG

29

( 2 ) INFORMATION FOR SEQ ID NO:101:

- ( i ) SEQUENCE CHARACTERISTICS:
- ( A ) LENGTH: 17 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:101:

ATTAACCCTC ACTAAAG

17

( 2 ) INFORMATION FOR SEQ ID NO:102:

- ( i ) SEQUENCE CHARACTERISTICS:
- ( A ) LENGTH: 19 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:102:

AATACGACTC ACTATAGGG

19

( 2 ) INFORMATION FOR SEQ ID NO:103:

- ( i ) SEQUENCE CHARACTERISTICS:
- ( A ) LENGTH: 34 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:103:

-continued

CCTCGAGCTC GAGCTTGGGT GGCTTTGGGG CATG

3 4

( 2 ) INFORMATION FOR SEQ ID NO:104:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 17 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:104:

ATTACCCCTC ACTAAAG

1 7

( 2 ) INFORMATION FOR SEQ ID NO:105:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 44 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:105:

CCCTCGAGCT CGAGGGGTCA CTGAGAACT AGAAAAAGAA TTAG

4 4

( 2 ) INFORMATION FOR SEQ ID NO:106:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 37 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:106:

CCGCGGCCGC GTATCTGTGG GAGCCTCAAG GGAGAAC

3 7

( 2 ) INFORMATION FOR SEQ ID NO:107:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 44 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:107:

CGCGCGGGCC CTGTGACATT GAATAGAGTG AGGGTCCTGT TGGG

4 4

( 2 ) INFORMATION FOR SEQ ID NO:108:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 45 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:108:

AAAGGTTTCA CATTGTAGC TTGCTGTGTC ATTGCGATCT CTACG

4 5

( 2 ) INFORMATION FOR SEQ ID NO:109:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 45 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:109:

GTGGTCCTAA ATAGTTCACT CTATTCAATG TCACACTCGA GCCGG

4 5

## ( 2 ) INFORMATION FOR SEQ ID NO:110:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 33 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:110:

TATATTCTAG AGCAAGCAAC AGTTACTGCG ACG

3 3

## ( 2 ) INFORMATION FOR SEQ ID NO:111:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 33 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:111:

TATATATCGA TCCGAAGCGT AGAGTCACAC TTG

3 3

## ( 2 ) INFORMATION FOR SEQ ID NO:112:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 18 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:112:

TTAACTGTCA AAAGCCAC

1 8

## ( 2 ) INFORMATION FOR SEQ ID NO:113:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 68 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:113:

CGATGTGGCT TTTAGATGTT AAACCAGAGA AACACACGGA CTTCGGTCCG TGGTATATTA

6 0

GCTGGTAT

6 8

## ( 2 ) INFORMATION FOR SEQ ID NO:114:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 70 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:114:

CTAGATACCA GCTAATATAC CACGGACCGA AGTCCGTGTG TTTCTCTGGT TTAACATCTA

6 0

AAAGCCACAT

7 0

## ( 2 ) INFORMATION FOR SEQ ID NO:115:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 42 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:115:

TATATCTCGA GACCACCATG AGTGCTGTAA GTAATAGGAA GC

4 2

( 2 ) INFORMATION FOR SEQ ID NO:116:

- ( i ) SEQUENCE CHARACTERISTICS:
  - ( A ) LENGTH: 36 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:116:

TATATCTCGA GCTAGAAGGC AAACCTAACA CCCAAC

3 6

( 2 ) INFORMATION FOR SEQ ID NO:117:

- ( i ) SEQUENCE CHARACTERISTICS:
  - ( A ) LENGTH: 31 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:117:

TATATGGGCC CTACATGTCC CACTGTTCAA G

3 1

( 2 ) INFORMATION FOR SEQ ID NO:118:

- ( i ) SEQUENCE CHARACTERISTICS:
  - ( A ) LENGTH: 31 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:118:

TATATGGGCC CGTACGGAAG GAAAGAAGTC A

3 1

( 2 ) INFORMATION FOR SEQ ID NO:119:

- ( i ) SEQUENCE CHARACTERISTICS:
  - ( A ) LENGTH: 32 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:119:

TATATGGGCC CATTTTGGTT TTGCTATGCG TA

3 2

( 2 ) INFORMATION FOR SEQ ID NO:120:

- ( i ) SEQUENCE CHARACTERISTICS:
  - ( A ) LENGTH: 16 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:120:

TCTCTGTCCT CCATGA

1 6

( 2 ) INFORMATION FOR SEQ ID NO:121:

- ( i ) SEQUENCE CHARACTERISTICS:
  - ( A ) LENGTH: 66 base pairs
  - ( B ) TYPE: nucleic acid
  - ( C ) STRANDEDNESS: single
  - ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:121:

TCGAGTCATG GAGAGAGGAG AACCAGAGAA ACACACGGAC TTCGGTCCGT GGTATATTAC

6 0

-continued

CTGGAT

6 6

( 2 ) INFORMATION FOR SEQ ID NO:122:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 64 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:122:

CGATCCAGGT AATATACCAC GGACCGAAGT CCGTGTGTTT CTCTGGTTCT CCTCTCTCCA

6 0

TGAC

6 4

( 2 ) INFORMATION FOR SEQ ID NO:123:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 35 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:123:

GCCTCGAGAC AATGTACAGG ATGCAACTCC TGTCT

3 5

( 2 ) INFORMATION FOR SEQ ID NO:124:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 36 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:124:

GAATCGATTT ATCAAGTCAG TGTTGGAGAT GATGCT

3 6

( 2 ) INFORMATION FOR SEQ ID NO:125:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 31 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:125:

TATATGGGCC CATCGAGGTG AGAAAGAGGA C

3 1

( 2 ) INFORMATION FOR SEQ ID NO:126:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 31 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:126:

TATATGGGCC CTGTATCTGG CGGACCCGTG G

3 1

( 2 ) INFORMATION FOR SEQ ID NO:127:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 31 base pairs  
 ( B ) TYPE: nucleic acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:127:

-continued

TATATGGGCC CGCAGACAAG ACGCGGGCG C

3 1

( 2 ) INFORMATION FOR SEQ ID NO:128:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 24 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:128:

AUCUCUACGG UGUCCUAAA UAGU

2 4

We claim:

1. An alphavirus vector construct, comprising a promoter 5' of viral cDNA which initiates the synthesis of RNA from the viral cDNA by a process of in vitro transcription, followed by a 5' sequence which initiates transcription of alphavirus RNA, followed by a nucleotide sequence encoding alphavirus non-structural proteins, a viral junction region which has been inactivated such that viral transcription of a subgenomic fragment is prevented, an internal ribosome entry site or a sequence which promotes ribosome readthrough between adjacent reading frames, and an alphavirus RNA polymerase recognition sequence.

2. An alphavirus vector construct, comprising a promoter 5' of viral cDNA which initiates the synthesis of RNA from the viral cDNA by a process of in vitro transcription, followed by a 5' sequence which initiates transcription of alphavirus RNA, followed by a nucleotide sequence encoding alphavirus non-structural proteins, a viral junction region which has been modified such that viral transcription of a subgenomic fragment is reduced, an internal ribosome entry site or a sequence which promotes ribosome readthrough between adjacent reading frames, and an alphavirus RNA polymerase recognition sequence.

3. An alphavirus vector construct, comprising a promoter 5' of viral cDNA which initiates the synthesis of RNA from the viral cDNA by a process of in vitro transcription, followed by a 5' sequence which initiates transcription of alphavirus RNA, followed by a nucleotide sequence encoding alphavirus non-structural proteins, a First viral junction region which has been inactivated such that viral transcription of a subgenomic fragment is prevented, a second viral junction region which has been modified such that viral transcription of the subgenomic fragment is reduced, an internal ribosome entry site or a sequence which promotes ribosome readthrough between adjacent reading frames, and an alphavirus RNA polymerase recognition sequence.

4. An alphavirus cDNA vector construct comprising a promoter 5' of viral cDNA which initiates the synthesis of RNA from the viral EDNA within a cell, followed by a 5' sequence which initiates transcription of alphavirus RNA, followed by a nucleotide sequence encoding alphavirus non-structural proteins, a viral junction region consisting of (i) all active viral junction region, (ii) a viral junction region which has been modified such that viral transcription of a subgenomic fragment is reduced, and (iii) a viral junction region which has been inactivated such that viral transcription of a subgenomic fragment is prevented, and an alphavirus RNA polymerase recognition sequence.

5. An alphavirus cDNA vector construct comprising a promoter 5' of viral cDNA which initiates the synthesis of RNA from the viral cDNA within a cell, followed by a 5'

sequence which initiates transcription of alphavirus RNA, a nucleotide sequence encoding alphavirus non-structural proteins, a first viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, followed by a second viral junction region which has been modified such that viral transcription of the subgenomic fragment is reduced, and an alphavirus RNA polymerase recognition sequence.

6. A vector construct according to any one of claims 1 to 5, further comprising a polyadenylation sequence.

7. A vector construct according to any one of claims 1 to 5 wherein said alphavirus is selected from the group consisting of Venezuelan Equine Encephalitis, Ross River and Semliki Forest viruses.

8. A vector construct according to any one of claims 1 to 5 wherein said alphavirus is Sindbis virus.

9. A vector construct according to any one of claims 1 to 5, further comprising a selected heterologous nucleotide sequence.

10. A vector construct according to claim 9 wherein said vector construct contains a selected heterologous nucleotide sequence ranging in size from about 100 bases to about 8 kb.

11. A vector construct according to claim 9 wherein said selected heterologous nucleotide sequence is a sequence encoding a protein selected from the group consisting of IL-12, IL-15, and GM-CSF.

12. A vector construct according to claim 9 wherein said selected heterologous nucleotide sequence is IL-2.

13. A vector construct according to claim 9 wherein said selected heterologous nucleotide sequence is obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, HBV, HIV, HSV, FeLV, FIV, HTLV-I, HTLV-II, and CMV.

14. A vector construct according to claim 9 wherein said selected heterologous nucleotide sequence is obtained from a hepatitis C virus.

15. A vector construct according to claim 9 wherein said selected heterologous nucleotide sequence is selected from the group consisting of an antisense sequence, a non-coding sense sequence, and a ribozyme sequence.

16. A vector construct according to any one of claims 1 to 5 wherein said vector construct contains no alphavirus structural protein genes.

17. A vector construct according to any one of claims 1 to 5 wherein a selected heterologous nucleotide sequence is located downstream from said viral junction region.

18. A vector construct according to claim 2 or 5 wherein a selected heterologous nucleotide sequence is located downstream from said second viral junction region.

19. A vector construct according to claim 9 wherein said selected heterologous nucleotide sequence is located within a nucleotide sequence encoding alphavirus non-structural proteins.

20. A vector construct according to claim 1, 3, 4, or 5 wherein said inactivated viral junction region consists of the nucleotide sequence as shown in Sequence ID: No. 1, from nucleotide number 7579, to nucleotide 7597.

21. A vector construct according to claim 3 or 5 further comprising an adenovirus L3 gene or CMV H301 gene.

22. A vector construct according to claim 3 or 5 further comprising a non-alphavirus packaging sequence.

23. The vector construct according to claim 3 or 5 further comprising a 3' transcription termination site.

24. The vector construct according to claim 23 wherein said transcription termination site is a termination/polyadenylation sequence.

25. A recombinant alphavirus particle which, upon introduction into a BHK cell, produces an infected cell which is viable at least 72 hours after infection.

26. A recombinant alphavirus particle which, upon introduction into a BHK cell, produces an infected cell which is viable at least 72 hours after infection, said particle also carrying a vector construct which directs the expression of at least one antigen or modified form thereof in target cells infected with the alphavirus particle, wherein said antigen or modified form thereof stimulates an immune response within an animal.

27. A recombinant alphavirus particle according to claim 26 wherein the expressed antigen elicits an immune response selected from the group consisting of a cell-mediated immune response, a HLA Class I-restricted immune response, and a HLA Class II-restricted immune response.

28. A cell infected with a recombinant alphavirus particle according to any one of claims 25 to 27.

29. A cell according to claim 27 wherein said cell is a mammalian cell.

30. A packaging cell line which inducibly expresses alphavirus structural proteins, and which, upon introduction of an alphavirus vector construct, produces recombinant alphavirus particles.

31. A packaging cell line according to claim 30 derived from mammalian cells.

32. A packaging cell line according to claim 30 derived from mammalian cells.

33. A packaging cell line according to claim 32 derived from insect cells.

34. A packaging cell line according to claim 32 wherein said insect cells are mosquito cells.

35. A packaging cell line according to claim 30 wherein the packaging cell line, upon introduction of a vector construct, produces alphavirus particles which infect human cells.

36. A packaging cell line according to claim 30 wherein an alphavirus inhibitory protein is not produced.

37. A packaging cell line suitable for packaging and production of an alphavirus vector, wherein the packaging

cell line comprises an expression cassette which directs the expression of VSV-G.

38. A packaging cell line according to claim 37, further comprising an expression cassette which directs the expression of one or more alphavirus structural proteins.

39. A packaging cell line according to claims 30 or 37 wherein said cell line expresses a gene product which suppresses apoptosis.

40. The packaging cell according to claim 39 wherein said gene product is encoded by a gene selected from the group consisting of bcl-2 oncogene, adenovirus E1B gene encoding a 19-kD protein, herpes simplex virus type 1  $\gamma$ 34.5 gene, and AcMNPV baculovirus p35 gene.

41. An alphavirus producer cell line, comprising a packaging cell line according to claim 30, and an alphavirus vector construct or alphavirus cDNA vector construct, wherein said producer cell line produces recombinant alphavirus particles.

42. An alphavirus producer cell line according to claim 41 wherein said recombinant alphavirus particles infect human cells.

43. An alphavirus producer cell line according to claim 41 wherein said producer cell line inducibly produces recombinant alphavirus particles.

44. An alphavirus producer cell line according to claim 41 wherein said producer cell line produces recombinant alphavirus particles in response to a differentiation state of said producer cell line.

45. A producer cell line suitable for packaging and production of a recombinant alphavirus particle, wherein the producer cell line comprises an expression cassette which directs the expression of gag/pol, an expression cassette which directs the expression of env, and an alphavirus vector construct containing a retroviral packaging sequence.

46. A producer cell line suitable for packaging and production of a recombinant alphavirus particle, wherein the producer cell line comprises one or more expression cassettes which direct the expression of non-alphaviral structural proteins, and an alphavirus vector construct comprising a packaging sequence corresponding to a virus from which the non-alphaviral structural proteins are derived.

47. A method for producing recombinant alphavirus particles from a packaging cell line, the method comprising introducing an alphavirus vector construct into a packaging cell line according to any one of claims 30 to 41 by a process selected from the group consisting of (i) transfection of the packaging cell line with a eukaryotic layered vector initiation system, (ii) transfection of the packaging cell line with RNA transcribed in vitro from an alphavirus vector construct, and (iii) infection of the packaging cell line with recombinant alphavirus particles.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
CERTIFICATE OF CORRECTION

PATENT NO : 5,843,723  
DATED : Dec. 1, 1998  
INVENTOR(S) : Thomas W. Dubensky, Jr., et al.

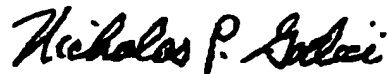
It is certified that errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the first line of the abstract, "method,, for" should read --method for--.  
Claim 3, column 213, line 45, "a First viral" should read --a first viral--.  
Claim 4, column 213, line 55, "EDNA" should read --cDNA--.  
Claim 20, column 215, line 3, "Sequence ID: No.1" should read --Sequence I.D. No. 1--.  
Claim 23, column 215, line 9, "claim 3 or 5 further" should read, --claim 3 or 5, further--.  
Claim 27, column 215, line 26, "clicits" should read --elicits--.  
Claim 32, column 215, line 42, "mammalian" should read --non-mammalian--.  
Claim 47, column 216, line 46, "claims 30 to 41" should read --claims 30 to 40--.

Signed and Sealed this

Twenty-seventh Day of March, 2001

Attest:



NICHOLAS P. GODICI

Attesting Officer

Acting Director of the United States Patent and Trademark Office



UNITED STATES PATENT AND TRADEMARK OFFICE  
CERTIFICATE OF CORRECTION

PATENT NO. : 5,843,723 Page 1 of 1  
DATED : December 1, 1998  
INVENTOR(S) : Thomas W. Dubensky, Jr., John M. Polo, Carlos E. Ibanez, Stephen M.W. Chang,  
Douglas J. Jolly, David A. Driver, Barbara A. Belli

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 213, claim 4,

Line 59, "...of (1) [all] an active..."

Line 61, "...fragment is reduced, [and] or (iii)..."

Column 214, claim 7,

Line 29, "...of Venezuelan [E] equine [E] encephalitis..."

Column 214, claim 18,

Line 62, "...according to claim [2] 3 or 5..."

Column 25, claim 21,

Line 6, "...an adeno virus [L] E3..."

Column 215, claim 25,

Line 15, "...into [at] a BHK cell..."

Signed and Sealed this

Eleventh Day of December, 2001

Attest:

*Nicholas P. Godici*

Attesting Officer

NICHOLAS P. GODICI  
Acting Director of the United States Patent and Trademark Office



US005593972A

**United States Patent** [19]

Weiner et al.

[11] **Patent Number:** 5,593,972[45] **Date of Patent:** Jan. 14, 1997[54] **GENETIC IMMUNIZATION**[75] **Inventors:** David B. Weiner, Merion; William V. Williams; Bin Wang, both of Havertown, all of Pa.[73] **Assignees:** The Wistar Institute; The Trustees of the University of Pennsylvania, both of Philadelphia, Pa.[21] **Appl. No.:** 125,012[22] **Filed:** Sep. 21, 1993**Related U.S. Application Data**

[63] Continuation-in-part of Ser. No. 29,336, Mar. 11, 1993, abandoned, which is a continuation-in-part of Ser. No. 8,342, Jan. 26, 1993, abandoned.

[51] **Int. Cl.<sup>5</sup>** ..... A61K 45/05; A61K 48/00; A61K 31/00[52] **U.S. Cl.** ..... 514/44; 424/278.1; 514/615; 514/818[58] **Field of Search** ..... 435/320.1; 424/93.1, 424/93.2, 93.21, 278.1; 514/44, 615, 818[56] **References Cited****U.S. PATENT DOCUMENTS**

4,224,404	9/1980	Viza et al.	435/2
4,394,448	7/1983	Szoka, Jr. et al.	435/172.3
4,806,463	2/1989	Goodchild et al.	435/5
4,945,050	7/1990	Sanford et al.	435/172.1
5,017,487	5/1991	Stunnenberg et al.	435/172.3
5,036,006	7/1991	Sanford et al.	435/170.1
5,185,254	2/1993	Limmenbach	435/172.3
5,466,676	11/1995	Booth et al.	514/44

**FOREIGN PATENT DOCUMENTS**

WO90/11092	10/1990	WIPO
WO91/12329	8/1991	WIPO
WO93/17706	9/1993	WIPO
WO93/23552	11/1993	WIPO

**OTHER PUBLICATIONS**

F. D. Ledley (1991) *Human Gene Therapy* 2:77-83.  
 B. F. Haynes (1993) *Science* 260: 1279-1286.  
 A. Hoffenbach et al. (1989) *The Journal of Immunology* 142: 452-462.  
 D. Torpey et al. (1993) *Clinical Immunology and Immunopathology* 68(3): 263-272.  
 L. Butini et al. (1994) *J. Cell. Biochem. Suppl.* 18B: 147, Abstract J306.  
 A. Knuth et al. (1991) *Current Opinion in Immunology* 3:659-664.  
 B. Wang et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:4156-4160.  
 D. J. Wells (1993) *Febs Letters* 332 (1,2):179-182.  
 R. F. Garry et al. (1990) *Science* 250: 1127-1129.  
 A. M. Schultz et al., *AIDS* 7(suppl. 1):S161-S170 (1993).  
 V. Glaser, *Genetic Engineering News* 16(1):6 (1996).  
 H. N. Eisen, "Introduction to Immune Responses," in *Microbiology*, Bernard D. Davis et al., eds. Hagerstown: Harper & Row, Publishers, 1980, p. 294.

Aldovini et al., "Mutations of RNA and Protein Sequences Involved in Human Immunodeficiency Virus Type 1 Packaging Result in Production of Noninfectious Virus," *J. of Virology*, 64: 1920-1926, 1990.

Benvenisty et al., "Direct introduction of genes into rats and expression of the genes," *Proc. Natl. Acad. Sci. USA*, 83:9551-9555, 1986.

Brandsma et al., "Use of a rapid, efficient inoculation method to induce papillomas by cottontail rabbit papillomavirus DNA shows that the E7 gene is required," *Proc. Natl. Acad. Sci. USA*, 88:4816-4820, 1991.

Desrosiers, "HIV with Multiple Gene Deletions as a Live Attenuated Vaccine for AIDS," *AIDS Research and Human Retroviruses*, 8:411-421, 1992.

Friedmann et al., "Progress Toward Human Gene Therapy," *Science*, 244:1275-1281, 1989.

Kaneda et al., "Increased Expression of DNA Cointroduced with Nuclear Protein in Adult Rat Liver," *Science*, 243:375-378, 1989.

Nicolau et al., "In vivo expression of rat insulin after intravenous administration of the liposome-entrapped gene for rat insulin I," *Proc. Natl. Acad. Sci. USA*, 80:1068-1072, 1983.

Ronen et al., "Expression of wild-type and mutant p53 proteins by recombinant vaccinia viruses," *Nucleic Acids Research*, 20:3435-3441, 1992.

Schauer et al., "The N-Terminal Region of HIV-1 Integrase is Required for Integration Activity, but not for DNA-Binding," *Biochem. and Biophys. Res. Commun.*, 185:874-880, 1992.

Seeger et al., "The cloned genome of ground squirrel hepatitis virus is infectious in the animal," *Proc. Natl. Acad. Sci. USA*, 81:5849-5852, 1984.

Wu et al., "Receptor-mediated Gene Delivery and expression in Vivo," *J. of Biological Chemistry*, 263:14621-14624, 1988.

(List continued on next page.)

**Primary Examiner**—Mindy Fleisher**Assistant Examiner**—Johnny F. Railey, II**Attorney, Agent, or Firm**—Woodcock Washburn Kurtz Mackiewicz & Norris

[57]

**ABSTRACT**

Methods of prophylactic and therapeutic immunization of an individual against pathogen infection, diseases associated with hyperproliferative cells and autoimmune diseases are disclosed. The methods comprise the steps of administering to cells of an individual, a nucleic acid molecule that comprises a nucleotide sequence that encodes a protein which comprises at least one epitope that is identical or substantially similar to an epitope of a pathogen antigen, a hyperproliferative cell associated protein or a protein associated with autoimmune disease respectively. In each case, nucleotide sequence is operably linked to regulatory sequences to enable expression in the cells. The nucleic acid molecule is free of viral particles and capable of being expressed in said cells. The cells may be contacted with a cell stimulating agent. Methods of prophylactically and therapeutically immunizing an individual against HIV are disclosed. Pharmaceutical compositions and kits for practicing methods of the present invention are disclosed.

**9 Claims, 12 Drawing Sheets**

## OTHER PUBLICATIONS

- Yang et al., "In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment," *Proc. Natl. Acad. Sci. USA*, 87:9568-9572, 1990.
- Zelenin et al., "High-velocity mechanical DNA transfer of the chloramphenicolacetyl transferase gene into rodent liver, kidney and mammary gland cells in organ explants and in vivo," *FEBS Letts.*, 280:94-96, 1991.
- Acsadi et al., "Human dystrophin expression in mdx mice after intramuscular injection of DNA constructs," *Nature* 352:815-818, 1991.
- Anderson, W. French, "Prospects for Human Gene Therapy," *Science* 226:401-409, 1984.
- Anilionis et al., "Structure of the glycoprotein gene in rabies virus," *Nature* 294:275278, 1981.
- Benoit et al., "Destruction and regeneration of skeletal muscle after treatment with a local anaesthetic, bupivacaine (Marcaine®)," *J. Anat.* 107:547-556, 1970.
- Berman et al., "Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160," *Nature*, 345:622-625, 1990.
- Brigham et al., "Rapid Communication: In Vivo Transfection of Murine Lungs with a Functioning Prokaryotic Gene Using A Liposome Vehicle," *American Journal of the Medical Sciences*, 298:278-281, 1989.
- Chaudhary et al., "A rapid method of cloning functional variable-region antibody genes in *Escherichia coli* as single-chain immunotoxins," *Proc. Natl. Acad. Sci. USA*, 87:1066-1070, 1990.
- Chen et al., "HIV-1 gp41 contains two sites for interaction with several proteins on the helper T-lymphoid cell line, H9," *AIDS*, 6:533-539, 1992.
- Cheng-Mayer et al., "Human Immunodeficiency virus can productively infect cultured human glial cells," *Proc. Natl. Acad. Sci. USA*, 84:3526-3530, 1987.
- Crowe et al., "Improved cloning efficiency of polymerase chain reaction (PCR) products after proteinase K digestion," *Nucleic Acids Research*, 19:184, 1991.
- Desquenne-Clark et al., "T-cell receptor peptide immunization leads to enhanced and chronic experimental allergic encephalomyelitis," *Proc. Natl. Acad. Sci. USA*, 88:7219-7223, 1991.
- Di Fiore et al., "erbB-2 Is a Potent Oncogene When Over-expressed in NIH/3T3 Cells," *Science*, 237:178-182, 1987.
- Dubensky et al., "Direct transfection of viral and plasmid DNA into the liver of spleen of mice," *Proc. Natl. Acad. Sci. USA*, 81:7529-7533, 1984.
- Felgner et al., "Gene Therapeutics," *Nature*, 349:351-352, 1991.
- Fisher et al., "A molecular clone of HTLV-III with biological activity," *Nature*, 316:262-265, 1985.
- Fisher et al., "HIV infection is blocked in vitro by recombinant soluble CD4," *Nature*, 331:76-78, 1988.
- Goudsmit et al., "Human antibody response to a strain-specific HIV-1 gp120 epitope associated with cell fusion inhibition," *AIDS*, 2:157-164, 1988.
- Hahn et al., "Suppression of Murine Lupus Nephritis By Administration of an Anti-Idiotypic Antibody to Anti-DNA," *J. of Immunology*, 132:187-190, 1984.
- Hall-Craggs, E. C. B., "Rapid Degeneration and Regeneration of a Whole Skeletal Muscle Following Treatment with Bupivacaine (Marcaine)," *Experimental Neurology*, 43:349-358, 1974.
- Howley, Peter M., "Papillomavirinae and Their Replication," *Virology*, Chapter 58:1625-1650, 1990.
- Howell et al., "Limited T-cell receptor Beta-chain heterogeneity among interleukin 2 receptor-positive synovial T cells suggests a role for superantigen in rheumatoid arthritis," *Proc. Natl. Acad. Sci. USA*, 88:10921-10925, 1991.
- Israel et al., "Biological Activity of Polyoma Viral DNA in Mice and Hamsters," *J. of Virology*, 29:990-996, 1979.
- Klein et al., "Transformation of Microbes, Plants and Animals by Particle Bombardment," *BioTechnology*, 10:286-291, 1992.
- Koenig et al., "Detection of AIDS Virus in Macrophages in Brian Tissue from AIDS Patients with Encephalopathy," *Science*, 233:1089-1093, 1986.
- Kowalski et al., "Functional Regions of the Envelope Glycoprotein of Human Immunodeficiency Virus Type 1," *Science*, 237:1351-1355, 1987.
- Langlois et al., "The Ability of Certain HIV Vaccines to Provoke Reactions Against Normal Cells," *Science*, 255:292-293, 1992.
- Lasky et al., "Neutralization of the AIDS Retrovirus by Antibodies to a Recombinant Envelope Glycoprotein," *Science*, 233:209-212, 1986.
- Lasky et al., "Delineation of a Region of the Human Immunodeficiency Virus Type 1 gp120 Glycoprotein Critical for Interaction with the CD4 Receptor," *Cell*, 50:975-985, 1987.
- Letvin et al., "Risks of Handling HIV," *Nature*, 349:573, 1991.
- Maddon et al., "The T4 Gene Encodes the AIDS Virus Receptor and Is Expressed in the Immune System and the Brain," *Cell*, 47:333-348, 1986.
- Montefiori et al., "Evaluation of Antiviral Drugs and Neutralizing Antibodies to Human Immunodeficiency Virus by a Rapid and Sensitive Microtiter Infection Assay," *J. of Clinical Microbiology*, 26:231-235, 1988.
- Morgenstern et al., "Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line," *Nucl. Acids Res.*, 18:3587-3596, 1990.
- Nabel et al., "Site-Specific Gene Expression in Vivo by Direct Gene Transfer into the Arterial Wall," *Science*, 249:1285-1288, 1990.
- Nara, Peter L., "Quantitative Infectivity Syncytium-Forming Microassay," *Basic Virologic Techniques*, 77-86.
- Oksenberg et al., "Limited heterogeneity of rearranged T-cell receptor V alpha transcripts in brains of multiple sclerosis patients," *Nature*, 345:344-348, 1990.
- Osther et al., "Protective Humoral Immune Responses to the Human Immunodeficiency Virus Induced in Immunized Pigs: A Possible Source of Therapeutic Immunoglobulin Preparations," *Hybridoma*, 10:673-683, 1991.
- Osther et al., "The Quick Western Blot, A Novel Transportable 50-Minute HIV-1 Antibody Test," *Transplantation*, 47:834-838, 1989.
- Paliard et al., "Evidence for the Effects of a Superantigen in Rheumatoid Arthritis," *Science*, 253:325-329, 1991.
- Putney et al., "Development of an HIV Subunit Vaccine," *V International Conference on AIDS*, Quebec, Canada; Jun. 4-9, 1989.
- Schrier et al., "B- and T-Lymphocyte Responses to an Immunodominant Epitope of Human Immunodeficiency Virus," *J. of Virology*, 62:2531-2536, 1988.

- Sun et al., "Generation and Characterization of Monoclonal Antibodies to the Putative CD4-Binding Domain of Human Immunodeficiency Virus Type 1 gp120," *J. of Virology*, 63:3579-3585, 1989.
- Shah et al., "Papillomaviruses," *Virology*, Chapter 59:1651-1676, 1990.
- Seed et al., "Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure," *Proc. Natl. Acad. Sci. USA*, 84:3365-3369, 1987.
- Szala et al., "Molecular cloning of cDNA for the carcinoma-associated antigen GA733-2," *Proc. Natl. Acad. Sci. USA*, 87:3542-3546, 1990.
- Tang et al., "Genetic immunization is a simple method for eliciting an immune response," *Nature* 356:152-154, 1992.
- Teitelbaum et al., "In Vivo Effects of Antibodies Against a High Frequency Idiotype of Anti-DNA Antibodies in MRL Mice," 132:1282-1285, 1984.
- Thomason et al., "Stable incorporation of a bacterial gene into adult rat skeletal muscle in vivo," *Cell Physiol.*, 27:C578-581, 1990.
- Ugen et al., (1992) *Generation of Monoclonal Antibodies Against the Amino Region of gp120 Which Elicits Antibody Dependent Cellular Cytotoxicity*, Cold Spring Harbor Laboratory, 1992.
- Ulmer et al., "Heterologous Protection Against Influenza by Injection of DNA Encoding a Viral Protein," *Science*, 259:1745-1749, 1993.
- Vandenbark et al., "Immunization with a synthetic T-cell receptor V-region peptide protects against experimental autoimmune encephalomyelitis," *Nature*, 341:541-544, 1989.
- Vitadello et al., "Gene Transfer in Regenerating Muscle," *J. of Cellular Biochemistry*, Suppl. 17E:252, Mar. 29-Apr. 25, 1993.
- Weiner et al., "Non-CD4 Molecules on Human Cells Important in HIV-1 Cell Entry," *Vaccines*, 115-120, 1989.
- Will et al., "Cloned HBV DNA causes hepatitis in chimpanzees," *Nature*, 299:740-742, 1982.
- Williams et al., "Molecular Diagnosis of *Borrelia burgdorferi* Infection (Lyme Disease)," *DNA and Cell Biology*, 11:207-213, 1992.
- Williams et al., "Restricted Heterogeneity of T Cell Receptor Transcripts in Rheumatoid Synovium," *J. Clin. Invest.*, 90:326-333, 1992.
- Wolff et al., "Direct Gene Transfer into Mouse Muscle in Vivo," *Science*, 247:1465-1468, 1990.
- Wolff et al., "Conditions Affecting Direct Gene Transfer into Rodent Muscle In Vivo," *BioTechniques*, 11:474-485, 1991.
- Wucherpfennig et al., "Shared Human T Cell Receptor V Beta Usage to Immunodominant Regions of Myelin Basic Protein," *Science*, 248:1016-1019, 1990.
- Fleckenstein et al., "Tumour induction with DNA of oncogenic primate herpesviruses," *Nature*, 274:57-59, 1978.
- Boiron et al., "A Biological Property of Deoxyribonucleic Acid," *Discussion and Preliminary Reports*, 150-153, 1965.
- McCoy et al., "Human Colon Carcinoma Ki-ras2 Oncogene and Its Correspondence Proto-Oncogene," *Mol. and Cellular Biology*, 4:1577-1582, 1984.
- Rowe, et al., "Studies of Mouse Polyoma Virus Infection," U.S. Department of Health, Education and Welfare, 379-391, 1958.
- Orth et al., "Infectious and Oncogenic Effect of DNA Extracted from Cells Infected with Polyoma Virus," *P.S.E.B.M.*, 115:1090-1095, 1964.
- McCutchan et al., "Enhancement of the Infectivity of Simian Virus 40 Deoxyribonucleic Acid with Diethylaminoethyl-Dextran," *J. of the Nat. Cancer Institute*, 41:351-356, 1968.
- Mayne et al., "Tumour Induction by Simian Adenovirus SA7 DNA Fragments," *Nature New Biology*, 232:182-183, 1971.
- Sol et al., "Oncogenicity of SV40 DNA in the Syrian Hamster," *J. gen. Vir.* 37:635-638, 1977.
- Rowe et al., "B. Ecology of a Mouse Tumor Virus," *Perspectives in Virology*, 177-190.

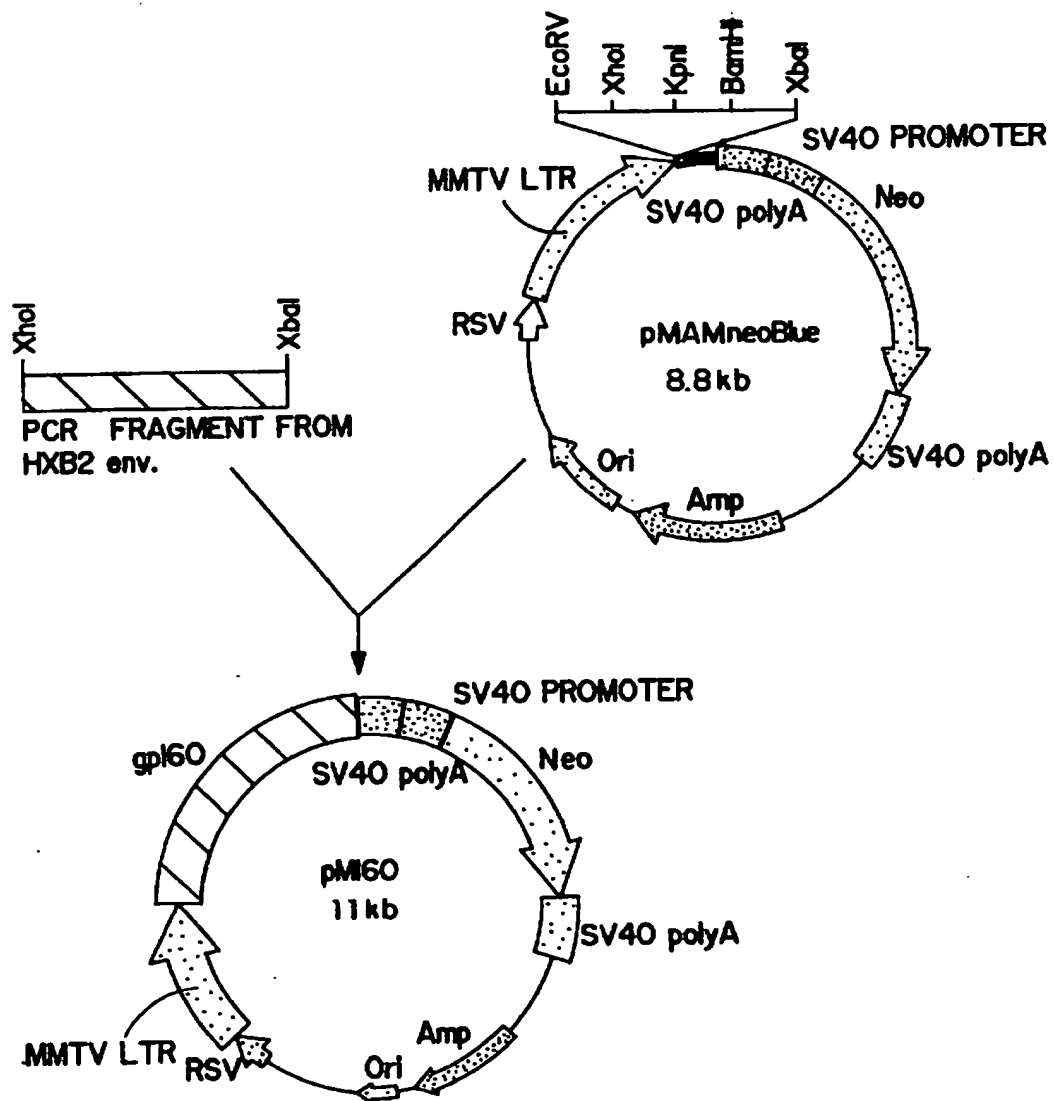


FIG. 1A

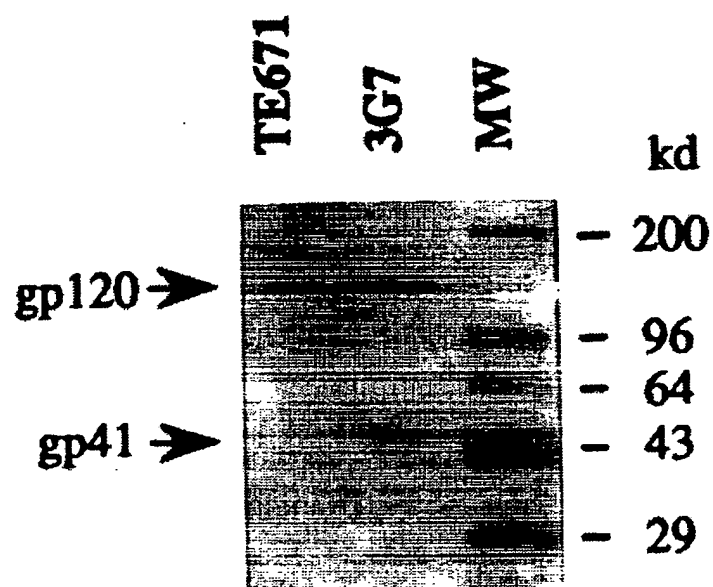


FIG.1B



FIG.2

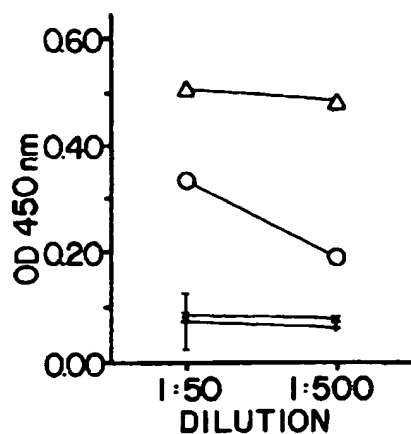


FIG. 3A

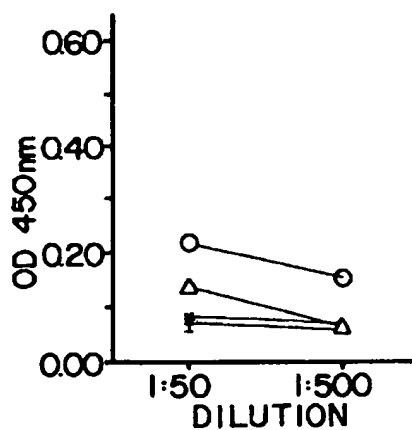


FIG. 3B

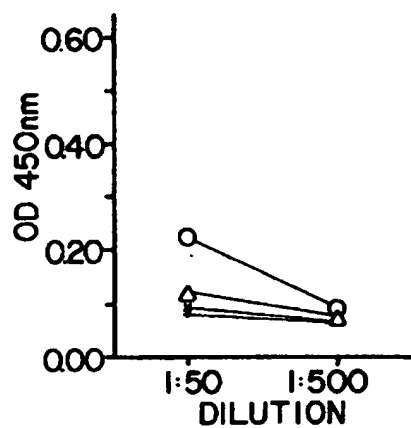


FIG. 3C

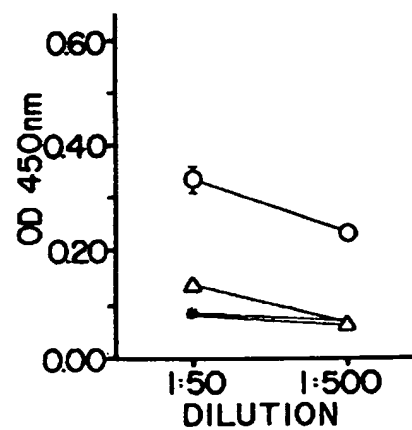


FIG. 3D

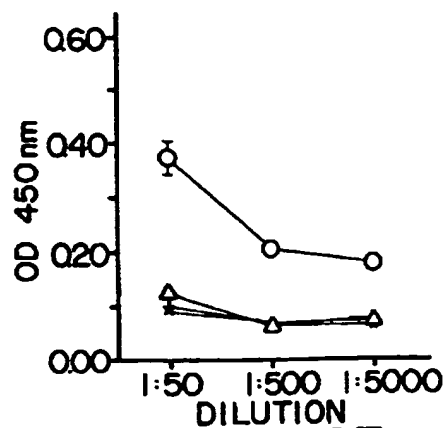


FIG. 3E



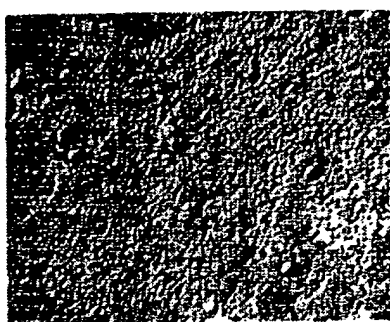


FIG.4A

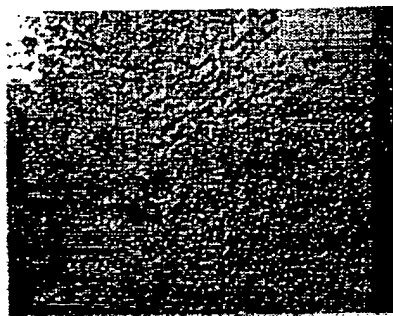


FIG.4B

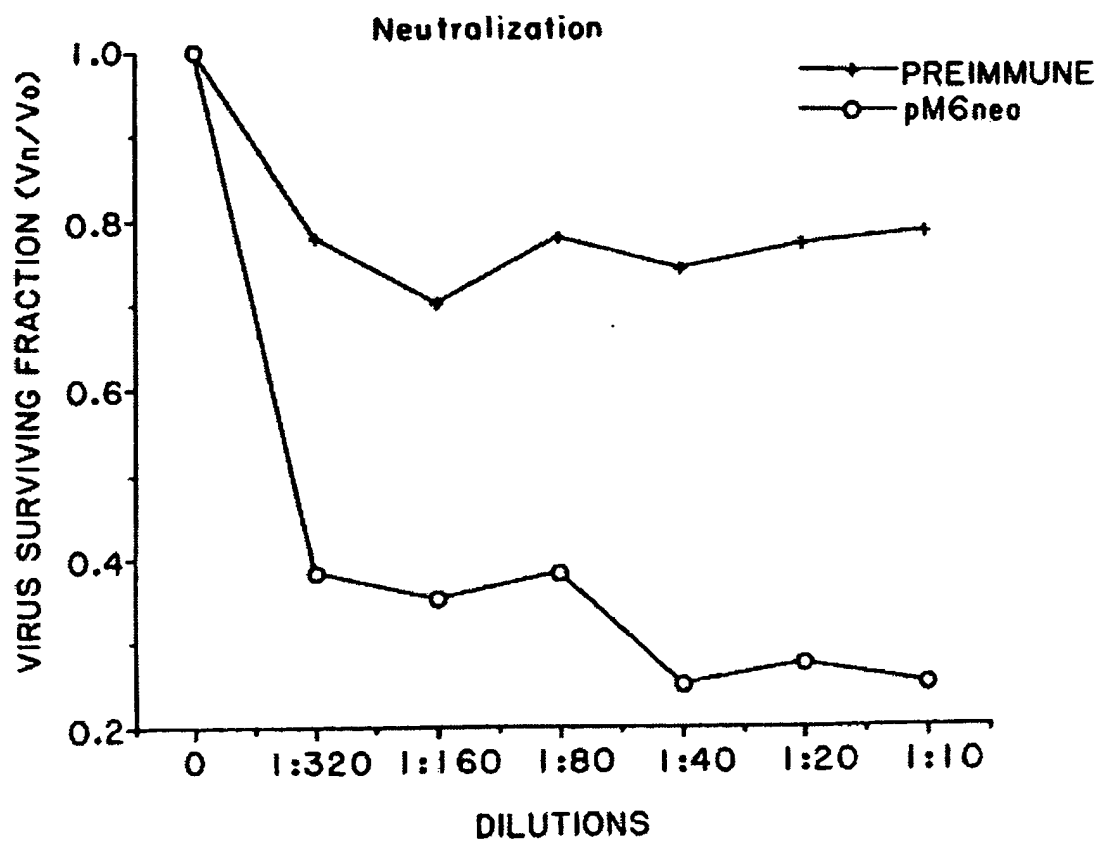


FIG.4C

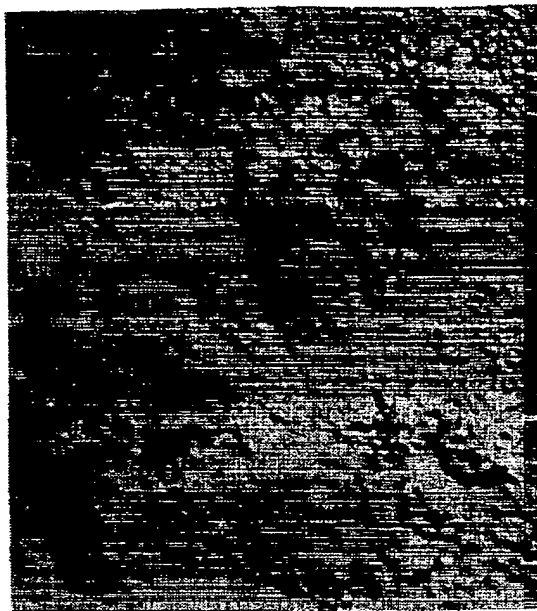


FIG.4D

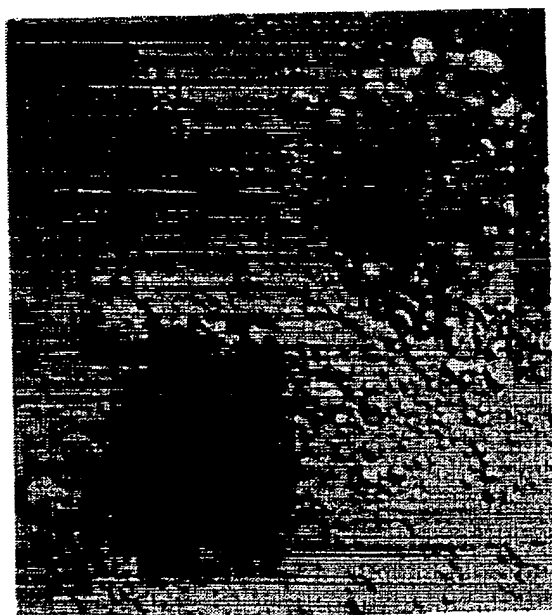


FIG.4E

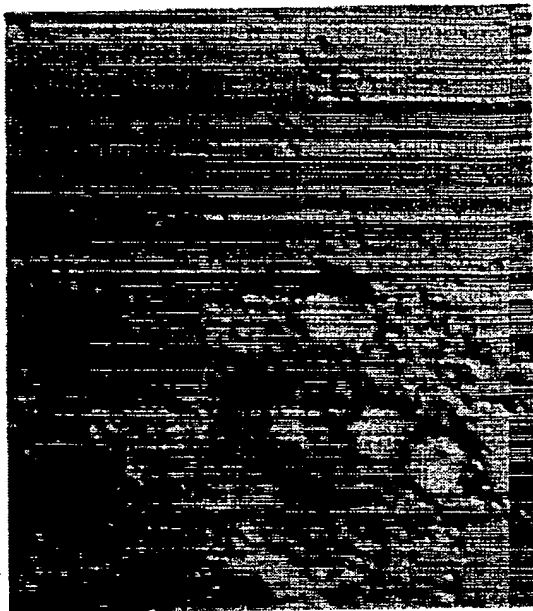


FIG. 4F

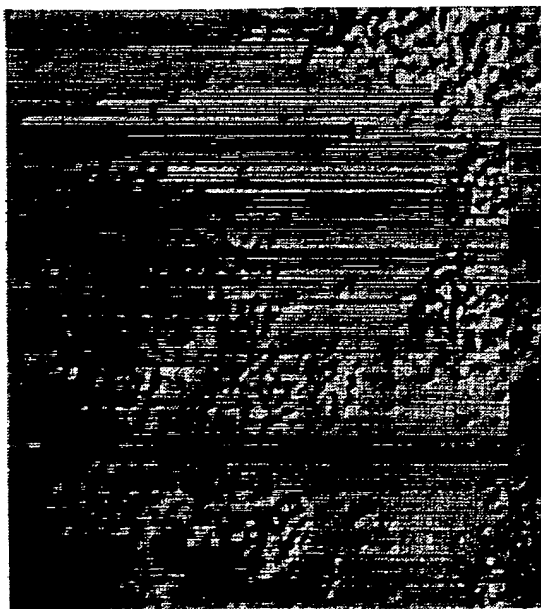


FIG. 4G

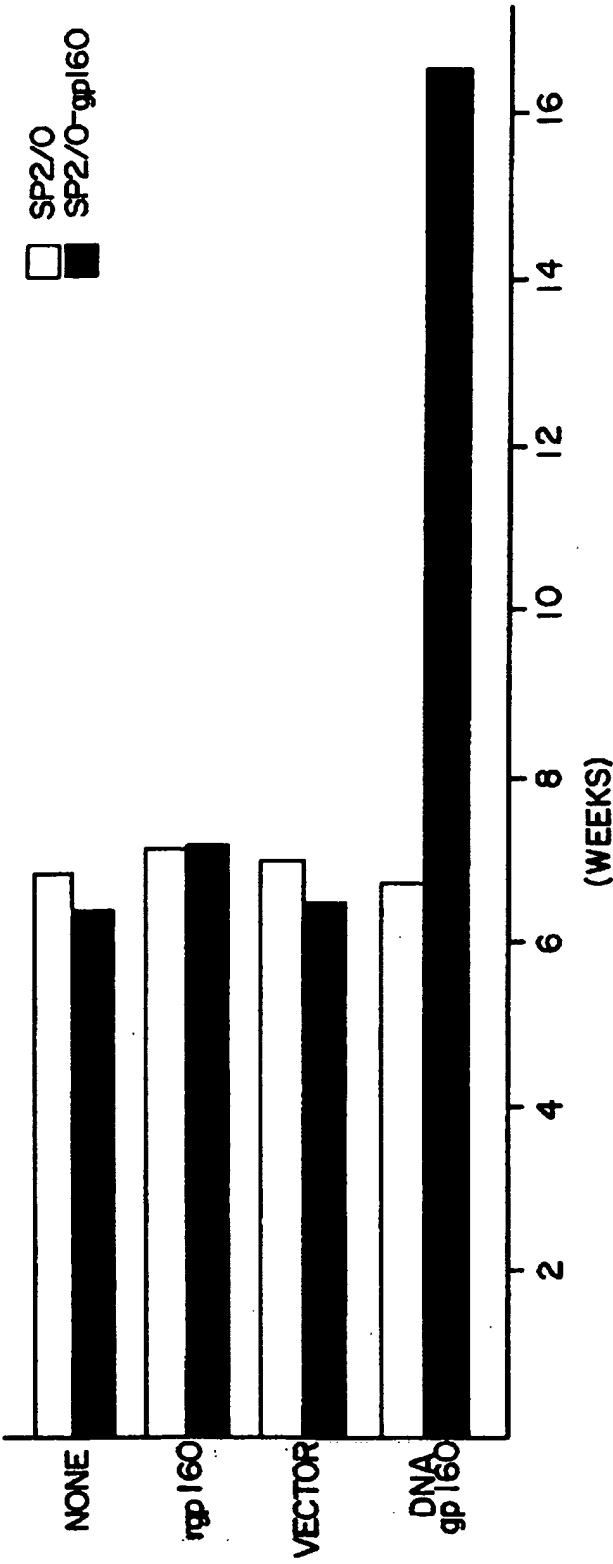


FIG. 5

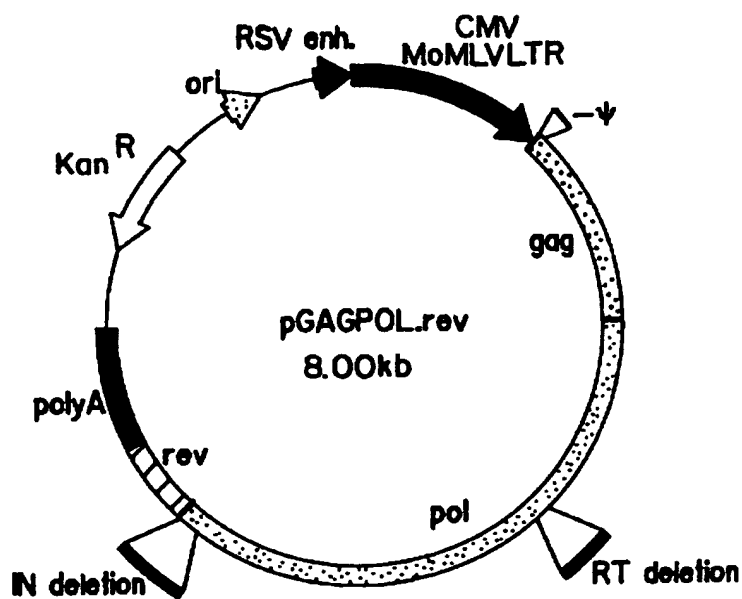


FIG. 6

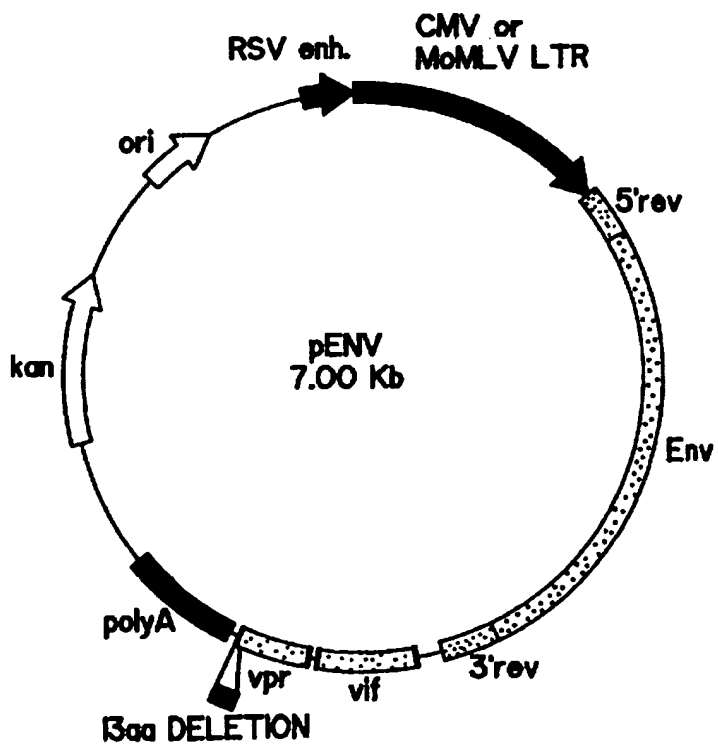


FIG. 7

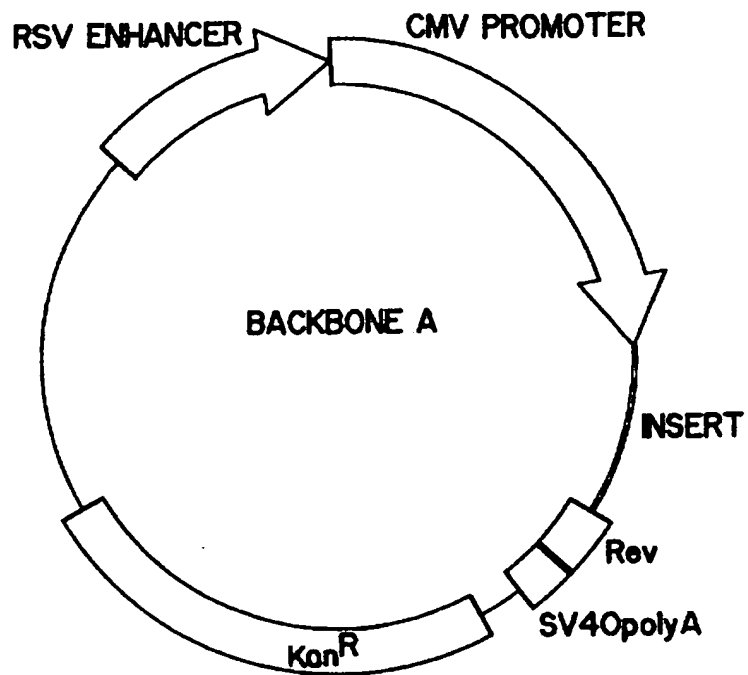


FIG. 8A

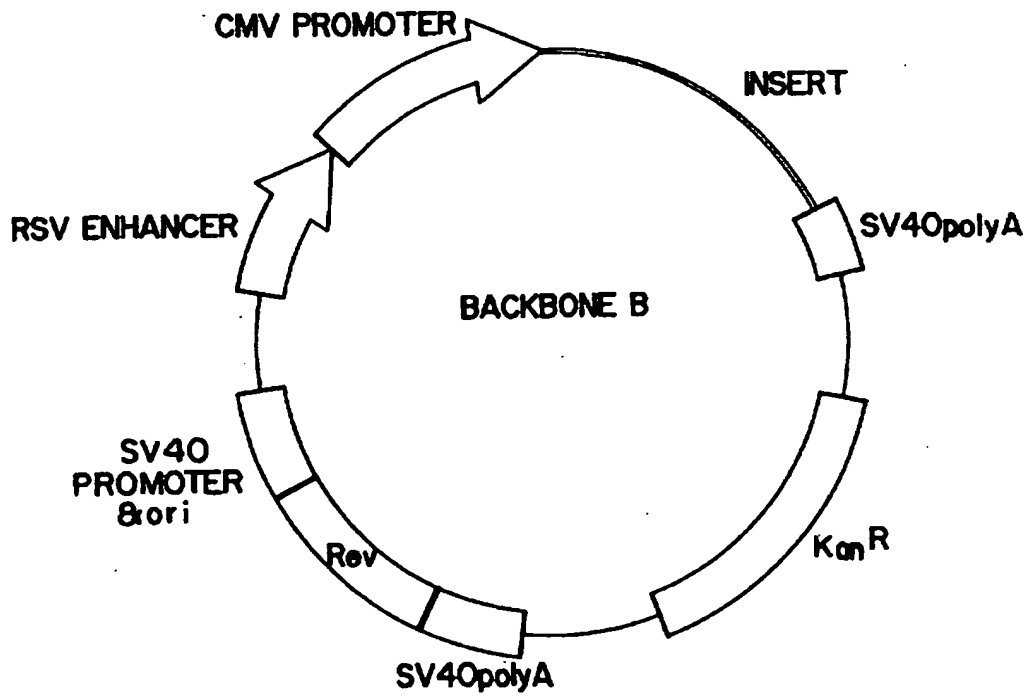


FIG. 8B

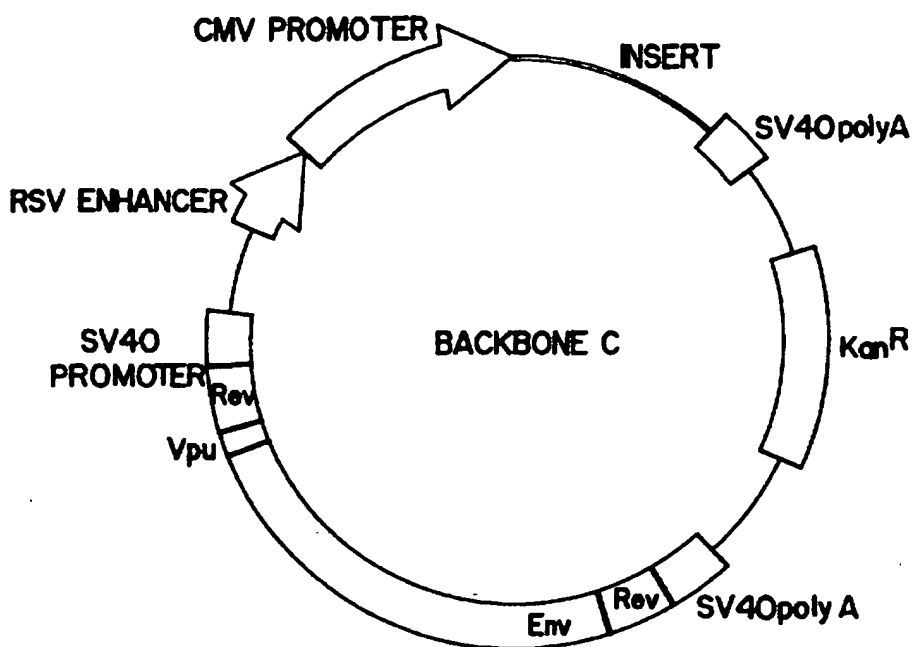


FIG. 8C

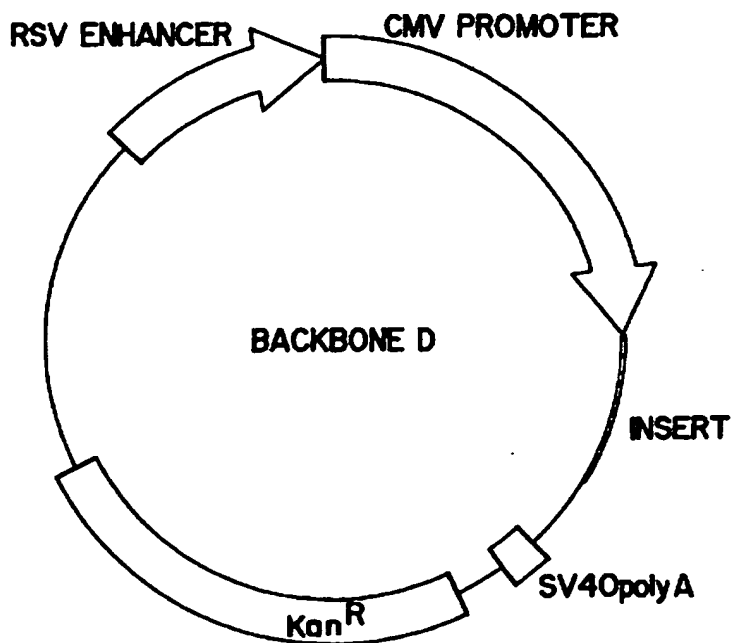


FIG. 8D

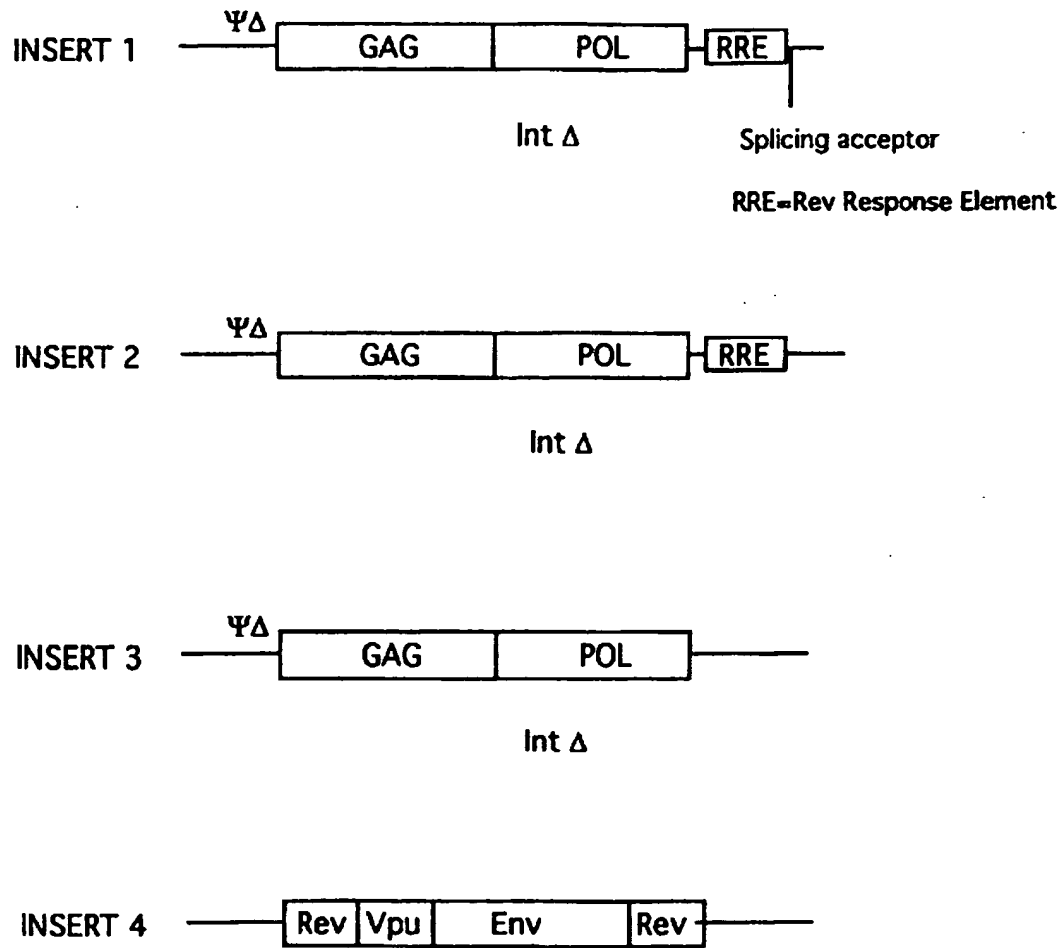


FIG. 9



1

## GENETIC IMMUNIZATION

## CROSS REFERENCE TO RELATED APPLICATIONS

This application is a Continuation-In-Part application of U.S. patent application Ser. No. 08/029,336 filed Mar. 11, 1993, now abandoned; which is a Continuation-In-Part application of U.S. patent application Ser. No. 08/008,342 filed Jan. 26, 1993, abandoned; both of which are incorporated herein by reference.

## FIELD OF THE INVENTION

The present invention relates to use of genetic material as immunizing agents. In particular, the present invention relates to the introduction of DNA molecules into an individual's tissues or cells that then can produce proteins capable of eliciting an immune response.

## BACKGROUND OF THE INVENTION

Vaccination and immunization generally refer to the introduction of a non-virulent agent against which an individual's immune system can initiate an immune response which will then be available to defend against challenge by a pathogen. The immune system identifies invading "foreign" compositions and agents primarily by identifying proteins and other large molecules which are not normally present in the individual. The foreign protein represents a target against which the immune response is made.

The immune system can provide multiple means for eliminating targets that are identified as foreign. These means include humoral and cellular responses which participate in antigen recognition and elimination. Briefly, the humoral response involves B cells which produce antibodies that specifically bind to antigens. There are two arms of the cellular immune response. The first involves helper T cells which produce cytokines and elicit participation of additional immune cells in the immune response. The second involves killer T cells, also known as cytotoxic T lymphocytes (CTLs), which are cells capable of recognizing antigens and attacking the antigen including the cell or particle it is attached to.

Vaccination has been singularly responsible for conferring immune protection against several human pathogens. In the search for safe and effective vaccines for immunizing individuals against infective pathogenic agents such as viruses, bacteria, and infective eukaryotic organisms, several strategies have been employed thus far. Each strategy aims to achieve the goal of protecting the individual against pathogen infection by administering to the individual, a target protein associated with the pathogen which can elicit an immune response. Thus, when the individual is challenged by an infective pathogen, the individual's immune system can recognize the protein and mount an effective defense against infection. There are several vaccine strategies for presenting pathogen proteins which include presenting the protein as part of a non-infective or less infective agent or as a discreet protein composition.

One strategy for immunizing against infection uses killed or inactivated vaccines to present pathogen proteins to an individual's immune system. In such vaccines, the pathogen is either killed or otherwise inactivated using means such as, for example, heat or chemicals. The administration of killed or inactivated pathogen into an individual presents the pathogen to the individual's immune system in a noninfective

2

form and the individual can thereby mount an immune response against it. Killed or inactivated pathogen vaccines provide protection by directly generating T-helper and humoral immune responses against the pathogenic immunogens. Because the pathogen is killed or otherwise inactivated, there is little threat of infection.

Another method of vaccinating against pathogens is to provide an attenuated vaccine. Attenuated vaccines are essentially live vaccines which exhibit a reduced infectivity. Attenuated vaccines are often produced by passaging several generations of the pathogen through a permissive host until the progeny agents are no longer virulent. By using an attenuated vaccine, an agent that displays limited infectivity may be employed to elicit an immune response against the pathogen. By maintaining a certain level of infectivity, the attenuated vaccine produces a low level infection and elicits a stronger immune response than killed or inactivated vaccines. For example, live attenuated vaccines, such as the poliovirus and smallpox vaccines, stimulate protective T-helper, T-cytotoxic, and humoral immunities during their nonpathogenic infection of the host.

Another means of immunizing against pathogens is provided by recombinant vaccines. There are two types of recombinant vaccines: one is a pathogen in which specific genes are deleted in order to render the resulting agent non-virulent. Essentially, this type of recombinant vaccine is attenuated by design and requires the administration of an active, non-virulent infective agent which, upon establishing itself in a host, produces or causes to be produced antigens used to elicit the immune response. The second type of recombinant vaccine employs infective non-virulent vectors into which genetic material that encode target antigens is inserted. This type of recombinant vaccine similarly requires the administration of an active infective non-virulent agent which, upon establishing itself in a host, produces or causes to be produced, the antigen used to elicit the immune response. Such vaccines essentially employ infective non-virulent agents to present pathogen antigens that can then serve as targets for an anti-pathogen immune response. For example, the development of vaccinia as an expression system for vaccination has theoretically simplified the safety and development of infectious vaccination strategies with broader T-cell immune responses.

Another method of immunizing against infection uses subunit vaccines. Subunit vaccines generally consist of one or more isolated proteins derived from the pathogen. These proteins act as target antigens against which an immune response may be mounted by an individual. The proteins selected for subunit vaccine are displayed by the pathogen so that upon infection of an individual by the pathogen, the individual's immune system recognizes the pathogen and mounts a defense against it. Because subunit vaccines are not whole infective agents, they are incapable of becoming infective. Thus, they present no risk of undesirable virulent infectivity that is associated with other types of vaccines. It has been reported that recombinant subunit vaccines such as the hepatitis B surface antigen vaccine (HBsAg) stimulate a more specific protective T-helper and humoral immune response against a single antigen. However, the use of this technology to stimulate broad protection against diverse pathogens remains to be confirmed.

Each of these types of vaccines carry severe drawbacks which render them less than optimally desirable for immunizing individuals against a particular pathogen.

It has been observed that absent an active infection, a complete immune response is not elicited. Killed and inactivated

3

tivated vaccines, because they do not reproduce or otherwise undergo an infective cycle, do not elicit the CTL arm of the cellular immune response in most cases. Additionally, killed and inactivated vaccines are sometimes altered by the means used to render them inactivated. These changes can affect the immunogenicity of the antigens. Subunit vaccines, which are merely discreet components of a pathogen, do not undergo any sort of infective cycle and often do not elicit the CTL arm of the cellular immune response. Absent the CTL arm, the immune response elicited by either vaccine is often insufficient to adequately protect an individual. In addition, subunit vaccines have the additional drawback of being both expensive to produce and purify.

Attenuated vaccines, on the other hand, often make very effective vaccines because they are capable of a limited, non-virulent infection and result in immune responses involving a humoral response and both arms of the cellular immune response. However, there are several problems associated with attenuated vaccines. First, it is difficult to test attenuated vaccines to determine when they are no longer pathogenic. The risk of the vaccine being virulent is often too great to properly test for effective attenuation. For example, it is not practically possible to test an attenuated form of Human Immunodeficiency virus (HIV) to determine if it is sufficiently attenuated to be a safe vaccine. Secondly, attenuated vaccines carry the risk of reverting into a virulent form of the pathogen. There is a risk of infecting individuals with a virulent form of the pathogen when using an attenuated vaccine.

Recombinant vaccines require the introduction of an active infective agent which, in many cases, is undesirable. Furthermore, in cases where the recombinant vaccine is the result of deletion of genes essential for virulence, such genes must exist and be identified. In vaccines in which pathogen genes are inserted into infective non-virulent vectors, many problems exist related to the immune response elicited against the vector antigens. These problems negatively impact the immune response elicited against the target antigen. First, the recombinant vaccine introduces a great number of vector antigens against which the immune system also responds. Secondly, the vector can be used only once per individual since, after the first exposure, the individual will develop immunity to the vector. These problems are both present, for example, in recombinant vaccines that employ vaccinia vectors such as those disclosed in U.S. Pat. No. 5,017,487 issued May 21, 1991 to Stummenberg et al. This technology has not been universally successful against diverse pathogenic organisms. It is also complicated by the large amount of excess vaccinia antigens presented in the vaccinee. Once vaccinated with the vaccinia vector, the vaccinee cannot be effectively vaccinated again using the vaccinia vector.

Accordingly, the most effective vaccines for invoking a strong and complete immune response carry the most risk of harming the individual while the safer alternatives induce an incomplete, and therefore, less effective immune response. Furthermore, many subunit vaccines and recombinant vaccines using non-virulent vectors to produce target proteins are most useful if a single antigenic component can be identified which is singularly protective against live challenge by a pathogen. However, both technologies require that the protective component be identified. Such identification is often both laborious and time-consuming.

A distinct advantage would exist if there were a rapid system for directly testing subunit vaccination strategies without tissue culture and in the absence of excess vector antigens. Furthermore, it would be particularly advanta-

4

geous if such a system could deliver an antigen that could be presented for development of both T cell immune arms.

There is a need for a means to immunize individuals against pathogen infection which can elicit a broad, biologically active protective immune response without risk of infecting the individual.

HIV infection represents a great threat to the human population today. Despite the intense resources expended and efforts made to develop an effective vaccine, the problem remains intractable. No vaccine is currently available that protects an individual against HIV infection. There is a great need for a method of immunizing an individual against HIV infection. There is a great need for an effective immunotherapy method to combat the development of AIDS in HIV infected individuals.

In addition to immunizing against pathogens, work has recently been undertaken to develop vaccines against cancer. Cancer vaccines currently being studied are essentially analogous to anti-pathogen subunit vaccines. Anti-cancer subunit vaccines essentially introduces a cancer-associated target protein into an individual. An immune response is elicited against the target protein in the same manner an immune response is elicited against a pathogen protein in the individual. The target protein is a protein that is specific to cancer cells. Subsequent appearance of the target protein when cancer occurs provides an immunogenic target for an immune response. Thus, the cancer vaccine immunizes an individual against cancer cells, an "endogenous pathogen", by immunizing against a target antigen specifically associated with the cancer. Specific proteins are administered which represent targets for an immunological response. As in the case of anti-pathogen subunit vaccines, the immune response elicited is often incomplete and insufficient to protect the individual. In particular, administration of a protein or peptide does not elicit a CTL response.

There is a need for an effective means to immunize individuals against hyperproliferative disease such as cancer in order to provide individuals with broad, biologically active protective immunity against specifically targeted hyperproliferating cells.

Many autoimmune diseases are mediated by specific antigen receptors. Autoimmune diseases generally refer to those diseases involving a self-directed immune response. Autoimmune diseases are referred to as being B cell mediated or T cell mediated. For example, Systemic Lupus Erythematosus (SLE) is considered a B cell mediated autoimmune disease. Many of the clinical manifestations of SLE are believed to be due to the presence of anti-DNA antibodies in the patients' serum, which combine with the antigen to form immune complexes. These immune complexes are deposited in tissues, setting off the inflammatory cascade. Rheumatoid Arthritis (RA) is an example of T cell mediated autoimmune disease. RA is believed to be mediated by autoreactive T cells present in the synovium (joint tissue), where they respond to an unknown antigen in the context of class II major histocompatibility complex (MHC II) molecules, such as HLA-DR4 which is genetically linked to RA. These T cells recognize a specific antigen associated with MHC II via their T cell antigen receptors (TCRs). Thus, autoreactive antigen receptors, such as antibodies or T cell antigen receptors are responsible for the initial recognition event in a series of pathogenic, inflammatory events which culminates in the clinical manifestations of autoimmune diseases such as SLE and RA.

Several studies have been performed in experimental systems where such autoreactive antigen receptors have

been targeted or deleted. Animal model systems for autoimmune disease include a murine lupus model which occurs in a strain of NZB/NZW mice, and an experimental allergic encephalomyelitis (EAE) model which can be produced in susceptible mouse and rat strains following inoculation with myelin basic protein (MBP). In murine SLE, anti-idiotypic antibodies have been used therapeutically in an attempt to delete the autoreactive B cells which produce the autoreactive antibodies. In some cases, these anti-idiotypic antibodies have improved clinical manifestations of the disease (Hahn, B. H. and F. M. Ebling, 1984 *J. Immunol.* 132(1):187-190), while in others they have worsened disease (Teitelbaum, D. et al., 1984 *J. Immunol.* 132(3):1282-1285). Similarly, in EAE, antibodies to autoreactive T cell antigen receptors have been utilized, as has been immunization with T cell antigen receptor-derived peptides. Again, in some instances this improves the disease (Vandenbark, A., et al., 1989 *Nature* 341:541-544, while in other worsening of the disease occurs (Desquerne-Clark, L., et al., 1990 *Proc. Natl. Acad. Sci. USA* 88:7219-7223).

Thus, while it is possible to vaccinate against autoimmune disease in some cases, the nature of the immune response elicited affects the clinical outcome of such therapies. For example, if the vaccination results in development of an antibody response, with subsequent anti-idiotypic development, these anti-idiotypic antibodies could target the autoreactive B cells or T cells for complement-mediated lysis, with resulting clinical improvement. Alternatively, if the immunization results in production of non-complement fixing anti-idiotypic antibodies, these would bind to the autoreactive B cells or T cells and cross-link their antigen receptors. Typically, this leads to activation of the cells and subsequent increased production of the autoreactive antibodies or T cells, with worsening of the clinical condition. Alternatively, if a predominant T cell response is elicited by vaccination, this could result in either a helper T cell response which would be expected to worsen disease or a killer/suppressor cell response which should improve the disease.

There is a need for an effective means to immunize individuals against and treat individuals suffering from autoimmune diseases which would elicit a CTL response capable of targeting either B cells that produce the antibodies involved in the disease (in the case of B cell mediated autoimmune disease) or the T cells that produce the specific T cell antigen receptor which are involved in the disease (in the case of T cell mediated autoimmune disease).

The direct introduction of a normal, functional gene into a living animal has been studied as a means for replacing defective genetic information. In such studies, DNA is introduced directly into cells of a living animal. Nabel, E. G., et al., (1990) *Science* 249:1285-1288, disclose site-specific gene expression in vivo of a beta-galactosidase gene that was transferred directly into the arterial wall in mice. Wolfe, J. A. et al., (1990) *Science* 247:1465-1468, disclose expression of various reporter genes that were directly transferred into mouse muscle in vivo. The use of direct gene transfer as an alternative anti-pathogen vaccination method is suggested. Acsadi G., et al., (1991) *Nature* 352:815-818, disclose expression of human dystrophin gene in mice after intramuscular injection of DNA constructs. Wolfe, J. A., et al., 1991 *BioTechniques* 11(4):474-485, which is incorporated herein by reference, refers to conditions affecting direct gene transfer into rodent muscle in vivo. Multiple injections of plasmid DNA are reported to result in higher levels of protein production but not to the extent that the levels of protein production are proportional to additional plasmid DNA added. Felgner, P. L. and G. Rhodes, (1991)

*Nature* 349:351-352, disclose direct delivery of purified genes in vivo as drugs without the use of retroviruses. Use of direct gene transfer by single injection are suggested as a possible vaccination strategy and a cellular immune response to HIV gp120 resulting from introduction of plasmid DNA encoding the same into cells is reported to have been observed. PCT International Application Number PCT/US90/01515 published Oct. 4, 1990 discloses methods of immunizing an individual against pathogen infection by directly injecting naked polynucleotides into the individual's cells in a single step procedure. The use of transfecting agents other than lipofectins is specifically excluded from the disclosed methods. The stimulation of inoculated cells is neither disclosed nor suggested. An HIV vaccine is disclosed which consists of the introduction of polynucleotides that encode the viral protein gp120. The operability of this vaccine is not evidenced. Thomason, D. B. et al., (1990) *Cell Physiol.* 27:C578-581 and PCT patent application Ser. No. WO 91/12329 disclose administering bupivacaine to muscle cells in order to induce satellite cell proliferation. In particular, Thomason, D. B. et al., (1990) *Cell Physiol.* 27:C578-581 and PCT patent application Ser. No. WO 91/12329 disclose retroviral-mediated transfer of genes into adult tissue in which a mitotically-active state of satellite cells is induced. The retroviruses contain recombinant retroviral RNA that includes a foreign reporter gene incorporated within the viral particle.

#### SUMMARY OF THE INVENTION

The present invention relates to a method of immunizing an individual against a pathogen. The method comprises the steps of contacting cells of said individual with an agent that facilitates the uptake of DNA by the cells, the agent preferably being a cell stimulating agent, and administering to the cells, a DNA molecule that comprises a DNA sequence that encodes a peptide which comprises at least an epitope identical or substantially similar to an epitope displayed on a pathogen antigen operatively linked to regulatory sequences. The DNA molecule is capable of being expressed in the cells of the individual.

The present invention relates to a method of immunizing a human against HIV. The method comprises the steps of administering to a human a DNA molecule that comprises a DNA sequence that encodes at least one peptide that comprises at least one epitope identical or substantially similar to an epitope displayed on an HIV protein operatively linked to regulatory sequences.

The present invention relates to a method of immunizing a human against HIV. The method comprises the steps of administering two different DNA molecules to different cells of the human. Each DNA molecule comprises a DNA sequence that encodes at least one peptide which comprises at least one epitope identical or substantially similar to an epitope displayed on an HIV protein operatively linked to regulatory sequences. The different DNA molecules are each capable of being expressed in human cells. The different DNA molecules comprise different DNA sequences that encode at least one different peptide from the other.

The present invention related to a method of immunizing an individual against a hyperproliferative disease. The method comprises the steps of administering to cells of an individual, a DNA molecule that comprises a DNA sequence that encodes a peptide that comprises at least an epitope identical or substantially similar to an epitope displayed on a hyperproliferative disease-associated protein operatively

linked to regulatory sequences; the DNA molecule being capable of being expressed in the cells.

The present invention relates to a method of immunizing an individual against an autoimmune disease. The method comprises the steps of administering to cells of an individual, a DNA molecule that comprises a DNA sequence that encodes a peptide that comprises at least an epitope identical or substantially similar to an epitope displayed on an autoimmune disease-associated protein operatively linked to regulatory sequences; the DNA molecule being capable of being expressed in the cells.

The present invention relates to an HIV vaccine comprising a pharmaceutically acceptable carrier or diluent and a DNA molecule that encodes one or more peptides that each comprises at least an epitope identical or substantially similar to an epitope displayed on at least one HIV protein operatively linked to regulatory sequences; the DNA molecule being capable of being expressed in human cells.

The present invention relates to an HIV vaccine comprising two inoculants. The first inoculant comprises a pharmaceutically acceptable carrier or diluent and a first DNA molecule. The first DNA molecule comprises a DNA sequence that encodes one or more peptides that each comprises at least an epitope identical or substantially similar to an epitope displayed on at least one HIV protein operatively linked to regulatory sequences; the DNA molecule being capable of being expressed in human cells. The second inoculant comprises a pharmaceutically acceptable carrier or diluent and a second DNA molecule. The second DNA molecule comprises a DNA sequence that encodes one or more peptides that each comprises at least an epitope identical or substantially similar to an epitope displayed on at least one HIV protein operatively linked to regulatory sequences; the DNA molecule being capable of being expressed in human cells. The first and second DNA molecules are different and encode different peptides.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a diagram depicting the construction of plasmid pM160 which was produced by inserting a PCR-generated fragment that encodes the HIV-HXB2 glycoprotein gp160 into plasmid pMAMneoBlue (Clontech).

FIG. 1B is a photograph of an autoradiogram of a Western blot of whole cell lysates of cells transfected with the pM160 plasmid (3G7 cells) versus vector-alone transfected cells (TE671 cells) showing production of gp120 and gp41 in 3G7 cells and not in TE671 cells.

FIG. 2 is a photograph of an autoradiogram showing immunoprecipitations of serum antibodies binding to  $^{125}$ I-gp160.

FIGS. 3A-3E are graphs showing ELISA results binding different sera to various proteins immobilized on microtiter plates.

FIGS. 4A and 4B are photographs of MT-2 cells infected with TCID<sub>50</sub> HIV-1/III<sub>B</sub> cell-free virus that was preincubated with serial dilutions of antisera.

FIG. 4C is a graph illustrating the neutralization values ( $V_{50}/V_0$ ) versus the dilution factors from results using control serum (x=pMAMneoBlue vector-immunized mice) and test sera (O=pM160-immunized mice).

FIGS. 4D-4G are photographs of H9/III<sub>B</sub> cells used in experiments to examine syncytial inhibition using sera from immunized and control animals.

FIG. 5 is a chart depicting the survival of immunized and non-immunized mice challenged with HIV gp160-labelled

and unlabelled tumor cells. Mice were immunized with recombinant gp160 protein, vector DNA only or recombinant vector comprising DNA encoding gp160. SP2/0 tumor cells or SP2/0-gp160 (SP2/0 cells transfected with DNA encoding gp160 and expressing gp160) tumor cells were introduced into the mice.

FIG. 6 is a plasmid map of pGAGPOL.rev.

FIG. 7 is a plasmid map of pENV.

FIG. 8 shows four backbones, A, B, C and D, used to prepare genetic construct.

FIG. 9 shows four inserts, 1, 2, 3 and 4 which are inserted into backbones to produce genetic constructs.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods of eliciting immune responses in an individual which can protect an individual from pathogen infection or combat diseases and disorders involving cells that produce specific proteins. According to the present invention, genetic material that encodes an immunogenic peptide or protein is directly administered to an individual either in vivo or to the cells of an individual ex vivo. The genetic material encodes a peptide or protein that shares at least an epitope with an immunogenic protein to be targeted. The genetic material is expressed by the individual's cells to form immunogenic target proteins that elicit an immune response. The resulting immune response is broad based: in addition to a humoral immune response, both arms of the cellular immune response are elicited. Thus, the immune responses elicited by vaccination methods of the present invention are particularly effective to protect against pathogen infection or combat cells associated with hyperproliferative diseases or autoimmune diseases.

The immune response elicited by the target protein that is produced by vaccinated cells in an individual is a broad-based immune response which involves B cell and T cell responses including cytotoxic T cell (CTL) responses. The target antigens produced within the cells of the host are processed intracellularly: broken down into small peptides, bound by Class I MHC molecules, and expressed on the cell surface. The Class I MHC-target antigen complexes are capable of stimulating CD8<sup>+</sup> T-cells, which are phenotypically the killer/suppressor cells. Genetic immunization according to the present invention is thus capable of eliciting cytotoxic T-cell (CTL) responses (killer cell responses). It has been observed that genetic immunization according to the present invention is more likely to elicit CTL responses than other methods of immunization.

The present invention is useful to elicit broad immune responses against a target protein. Target proteins may be proteins specifically associated with pathogens or the individual's own "abnormal" cells. The present invention is useful to immunize individuals against pathogenic agents and organisms such that an immune response against a pathogen protein provides protective immunity against the pathogen. The present invention is particularly useful to protect an individual against infection by non-encapsulated intracellular pathogens which produce proteins within the host cells. The immune response generated against such proteins is capable of eliminating infected cells using CTLs which specifically recognize and eliminate such infected cells. The CTL response is crucial in protection against pathogens such as viruses and other intracellular pathogens which produce proteins within infected cells. The present

invention is useful to combat hyperproliferative diseases and disorders such as cancer by eliciting an immune response against a target protein that is specifically associated with the hyperproliferative cells. In such cases, a cytotoxic immune response against the hyperproliferating cells which produce the target protein is elicited. The present invention is useful to combat autoimmune diseases and disorders by eliciting an immune response against a target protein that is specifically associated with cells involved in the autoimmune condition. In such cases, the cytotoxic immune response against cells that produce the target protein is provided. The CTL response can be utilized for the specific elimination of deleterious cell types which, during their production of proteins, display antigens bound by Class I MHC molecules. Therefore, genetic immunization according to the present invention is more likely to result in anti-pathogen protection and therapy than standard immunization using killed, inactivated or protein—or peptide-based subunit vaccines and furthermore, may be used in immunization procedures to protect against and treat individuals suffering from cancer and autoimmune diseases.

Genetic immunization according to the present invention elicits an effective immune response without the use of infective agents or infective vectors. Vaccination techniques which usually do produce a CTL response do so through the use of an infective agent. A complete, broad based immune response is not generally exhibited in individuals immunized with killed, inactivated or subunit vaccines. The present invention achieves the full complement of immune responses in a safe manner without the risks and problems associated with vaccinations that use infectious agents.

According to some embodiments of the present invention, cells are treated with compounds that facilitate uptake of genetic constructs by the cells. According to some embodiments of the present invention, cells are treated with compounds that stimulate cell division and facilitate uptake of genetic constructs. Administration of compounds that facilitate uptake of genetic constructs by the cells including cell stimulating compounds results in a more effective immune response against the target protein encoded by the genetic construct.

According to some embodiments of the present invention, the genetic construct is administered to an individual using a needleless injection device. According to some embodiments of the present invention, the genetic construct is simultaneously administered to an individual intradermally, subcutaneously and intramuscularly using a needleless injection device. Administration of genetic constructs using needleless injection devices is disclosed in U.S. patent application Ser. No. 08/093,235 filed Jul. 15, 1993, which is incorporated herein by reference.

According to the present invention, DNA or RNA that encodes a target protein is introduced into the cells of an individual where it is expressed, thus producing the target protein. The DNA or RNA is linked to regulatory elements necessary for expression in the cells of the individual. Regulatory elements for DNA include a promoter and a polyadenylation signal. In addition, other elements, such as a Kozak region, may also be included in the genetic construct.

As used herein, the term "genetic construct" refers to the DNA or RNA molecule that comprises a nucleotide sequence which encodes the target protein and which includes initiation and termination signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of the

vaccinated individual. As used herein, the term "expressible form" refers to gene constructs which contain the necessary regulatory elements operable linked to a coding sequence that encodes a target protein, such that when present in the cell of the individual, the coding sequence will be expressed. As used herein, the term "genetic vaccine" refers to a pharmaceutical preparation that comprises a genetic construct.

As used herein, the term "target protein" refers to a protein against which an immune response can be elicited. The target protein is an immunogenic protein which shares at least an epitope with a protein from the pathogen or undesirable cell-type such as a cancer cell or a cell involved in autoimmune disease against which immunization is required. The target protein is an immunogenic protein derived from the pathogen or undesirable cell-type such as a cancer cell or a cell involved in autoimmune disease. Target proteins share epitopes with either pathogen-associated proteins, proteins associated with hyperproliferating cells, or proteins associated with autoimmune disorders, depending upon the type of genetic vaccine. The immune response directed against the target protein will protect the individual against the specific infection or disease with which the target protein is associated. For example, a genetic vaccine with a DNA or RNA molecule that encodes a pathogen-associated target protein is used to elicit an immune response that will protect the individual from infection by the pathogen. Likewise, a genetic vaccine with a DNA or RNA molecule that encodes a target protein associated with a hyperproliferative disease such as, for example, a tumor-associated protein, is used to elicit an immune response directed at hyperproliferating cells. A genetic vaccine with a DNA or RNA molecule that encodes a target protein that is associated with T cell receptors or antibodies involved in autoimmune diseases is used to elicit an immune response that will combat the autoimmune disease by eliminating cells in which the natural form of target protein is being produced. Target proteins may be either pathogen-associated proteins, proteins associated with hyperproliferating cells, or proteins associated with autoimmune disorders, depending upon the type of genetic vaccine.

As used herein, the term "sharing an epitope" refers to proteins which comprise at least one epitope that is identical to or substantially similar to an epitope of another protein.

As used herein, the term "substantially similar epitope" is meant to refer to an epitope that has a structure which is not identical to an epitope of a protein but nonetheless invokes an cellular or humoral immune response which cross reacts to that protein.

The genetic construct of genetic vaccines comprise a nucleotide sequence that encodes a target protein operably linked to regulatory elements needed for gene expression. Accordingly, incorporation of the DNA or RNA molecule into a living cell results in the expression of the DNA or RNA encoding the target protein and thus, production of the target protein.

When taken up by a cell, the genetic construct which includes the nucleotide sequence encoding the target protein operably linked to the regulatory elements may remain present in the cell as a functioning extrachromosomal molecule or it may integrate into the cell's chromosomal DNA. DNA may be introduced into cells where it remains as separate genetic material in the form of a plasmid. Alternatively, linear DNA which can integrate into the chromosome may be introduced into the cell. When introducing DNA into

the cell, reagents which promote DNA integration into chromosomes may be added. DNA sequences which are useful to promote integration may also be included in the DNA molecule. Since integration into the chromosomal DNA necessarily requires manipulation of the chromosome, it is preferred to maintain the DNA construct as a replicating or non-replicating extrachromosomal molecule. This reduces the risk of damaging the cell by splicing into the chromosome without affecting the effectiveness of the vaccine. Alternatively, RNA may be administered to the cell. It is also contemplated to provide the genetic construct as a linear minichromosome including a centromere, telomeres and an origin of replication.

The necessary elements of a genetic construct of a genetic vaccine include a nucleotide sequence that encodes a target protein and the regulatory elements necessary for expression of that sequence in the cells of the vaccinated individual. The regulatory elements are operably linked to the DNA sequence that encodes the target protein to enable expression.

The molecule that encodes a target protein is a protein-encoding molecule which is translated into protein. Such molecules include DNA or RNA which comprise a nucleotide sequence that encodes the target protein. These molecules may be cDNA, genomic DNA, synthesized DNA or a hybrid thereof or an RNA molecule such as mRNA. Accordingly, as used herein, the terms "DNA construct", "genetic construct" and "nucleotide sequence" are meant to refer to both DNA and RNA molecules.

The regulatory elements necessary for gene expression of a DNA molecule include: a promoter, an initiation codon, a stop codon, and a polyadenylation signal. In addition, enhancers are often required for gene expression. It is necessary that these elements be operable in the vaccinated individual. Moreover, it is necessary that these elements be operably linked to the nucleotide sequence that encodes the target protein such that the nucleotide sequence can be expressed in the cells of a vaccinated individual and thus the target protein can be produced.

Initiation codons and stop codon are generally considered to be part of a nucleotide sequence that encodes the target protein. However, it is necessary that these elements are functional in the vaccinated individual.

Similarly, promoters and polyadenylation signals used must be functional within the cells of the vaccinated individual.

Examples of promoters useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV) as well as promoters from human genes such as human Actin, human Myosin, human Hemoglobin, human muscle creatine and human metallothionein.

Examples of polyadenylation signals useful to practice the present invention, especially in the production of a genetic vaccine for humans, include but are not limited to SV40 polyadenylation signals and LTR polyadenylation signals. In particular, the SV40 polyadenylation signal which is in pCEP4 plasmid (Invitrogen, San Diego Calif.), referred to as the SV40 polyadenylation signal, is used.

In addition to the regulatory elements required for DNA expression, other elements may also be included in the DNA

molecule. Such additional elements include enhancers. The enhancer may be selected from the group including but not limited to: human Actin, human Myosin, human Hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

Genetic constructs can be provided with mammalian origin of replication in order to maintain the construct extrachromosomally and produce multiple copies of the construct in the cell. Plasmids pCEP4 and pREP4 from Invitrogen (San Diego, Calif.) contain the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration.

An additional element may be added which serves as a target for cell destruction if it is desirable to eliminate cells receiving the genetic construct for any reason. A herpes thymidine kinase (tk) gene in an expressible form can be included in the genetic construct. When the construct is introduced into the cell, tk will be produced. The drug gancyclovir can be administered to the individual and that drug will cause the selective killing of any cell producing tk. Thus, a system can be provided which allows for the selective destruction of vaccinated cells.

In order to be a functional genetic construct, the regulatory elements must be operably linked to the nucleotide sequence that encodes the target protein. Accordingly, it is necessary for the initiation and termination codons to be in frame with the coding sequence.

In order to maximize protein production, regulatory sequences may be selected which are well suited for gene expression in the vaccinated cells. Moreover, codons may be selected which are most efficiently transcribed in the vaccinated cell. One having ordinary skill in the art can produce DNA constructs which are functional in vaccinated cells.

In order to test expression, genetic constructs can be tested for expression levels in vitro using tissue culture of cells of the same type as those to be vaccinated. For example, if the genetic vaccine is to be administered into human muscle cells, muscle cells grown in culture such as solid muscle tumors cells of rhabdomyosarcoma may be used as an in vitro model to measure expression level.

The present invention provides methods of conferring a broad based protective immune response against pathogen infection, hyperproliferative diseases and autoimmune diseases without the use of infectious agents. The genetic constructs used in the present invention are not incorporated with retroviral particles. The genetic constructs are taken up by the cell without viral particle-mediated insertion such as that which occurs when retrovirus particles with retroviral RNA that is incorporated in retroviral particles infects a cell. As used herein, the term "free from viral particles" is meant to refer to genetic constructs that are not incorporated within viral particles. In some embodiments, the genetic constructs constitute less than a complete, replicatable viral genome such that upon introduction into the cell, the genetic construct possesses insufficient genetic information to direct production of infectious viral particles. As used herein, the term "incomplete viral genome" is meant to refer to a genetic construct which contains less than a complete genome such that incorporation of such a genetic construct into a cell does not constitute introduction of sufficient genetic information for the production of infectious virus.

One aspect of the present invention provides a method of conferring a broad based protective immune response against pathogen infection, diseases associated with hyperproliferative cells or autoimmune diseases by administering

genetic constructs to cells contacted with an agent that facilitates the uptake of genetic material, particularly cell stimulating agents. The genetic construct may be administered with or without the use of microprojectiles.

The present invention may be used to immunize an individual against all pathogens such as viruses, prokaryotic and pathogenic eukaryotic organisms such as unicellular pathogenic organisms and multicellular parasites. The present invention is particularly useful to immunize an individual against those pathogens which infect cells and which are not encapsulated such as viruses, and prokaryotic such as gonorrhea, listeria and shigella. In addition, the present invention is also useful to immunize an individual against protozoan pathogens which include a stage in the life cycle where they are intracellular pathogens. As used herein, the term "intracellular pathogen" is meant to refer to a virus or pathogenic organism that, at least part of its reproductive or life cycle, exists within a host cell and therein produces or causes to be produced, pathogen proteins.

Table 1 provides a listing of some of the viral families and genera for which vaccines according to the present invention can be made. DNA constructs that comprise DNA sequences which encode the peptides that comprise at least an epitope identical or substantially similar to an epitope displayed on a pathogen antigen such as those antigens listed on the tables are useful in vaccines.

In addition to being particularly effective against pathogens which infect the cells of an individual, the present invention is also useful to immunize an individual against other pathogens including prokaryotic and eukaryotic protozoan pathogens as well as multicellular parasites. Table 2 contains a list of bacterial and eukaryotic pathogens for which vaccines according to the present invention may be made.

In order to produce a genetic vaccine to protect against pathogen infection, genetic material which encodes immunogenic proteins against which a protective immune response can be mounted must be included in the genetic construct. Whether the pathogen infects intracellularly, for which the present invention is particularly useful, or extracellularly, it is unlikely that all pathogen antigens will elicit a protective response. Because DNA and RNA are both relatively small and can be produced relatively easily, the present invention provides the additional advantage of allowing for vaccination with multiple pathogen antigens. The genetic construct used in the genetic vaccine can include genetic material which encodes many pathogen antigens. For example, several viral genes may be included in a single construct thereby providing multiple targets. In addition, multiple inoculants which can be delivered to different cells in an individual can be prepared to collectively include, in some cases, a complete or, more preferably, an incomplete such as a near complete set of genes in the vaccine. For example, a complete set of viral genes may be administered using two constructs which each contain a different half of the genome which are administered at different sites. Thus, an immune response may be invoked against each antigen without the risk of an infectious virus being assembled. This allows for the introduction of more than a single antigen target and can eliminate the requirement that protective antigens be identified.

The ease of handling and inexpensive nature of DNA and RNA further allow for more efficient means of screening for protective antigens. Genes can be sorted and systematically tested much more easily than proteins. The pathogenic agents and organism for which the vaccine is being produced

to protect against is selected and an immunogenic protein is identified. Tables 1 and 2 include lists of some of the pathogenic agents and organisms for which genetic vaccines can be prepared to protect an individual from infection by them.

Another aspect of the present invention provides a method of conferring a broad based protective immune response against hyperproliferating cells that are characteristic in hyperproliferative diseases and to a method of treating individuals suffering from hyperproliferative diseases. As used herein, the term "hyperproliferative diseases" is meant to refer to those diseases and disorders characterized by hyperproliferation of cells. Examples of hyperproliferative diseases include all forms of cancer and psoriasis.

It has been discovered that introduction of a genetic construct that includes a nucleotide sequence which encodes an immunogenic "hyperproliferating cell"-associated protein into the cells of an individual results in the production of those proteins in the vaccinated cells of an individual. As used herein, the term "hyperproliferative-associated protein" is meant to refer to proteins that are associated with a hyperproliferative disease. These proteins can elicit a broad biologically active immune response in the individual including CTLs that can effectively combat and eliminate hyperproliferating cells in the individual. Thus, to immunize against hyperproliferative diseases, a genetic construct that includes a nucleotide sequence which encodes a protein that is associated with a hyperproliferative disease is administered to an individual. When expressed, the protein produced elicits an immune response directed at cells that produce the protein.

In order for the hyperproliferative-associated protein to be an effective immunogenic target, it must be a protein that is produced exclusively or at higher levels in hyperproliferative cells as compared to normal cells. Target antigens include such proteins, fragments thereof and peptides which comprise at least an epitope found on such proteins. In some cases, a hyperproliferative-associated protein is the product of a mutation of a gene that encodes a protein. The mutated gene encodes a protein which is nearly identical to the normal protein except it has a slightly different amino acid sequence which results in a different epitope not found on the normal protein.

Such target proteins include those which are proteins encoded by oncogenes. Generally, oncogenes can be divided into three groups depending upon the portion of the cell where their gene products are found. Oncogenes such as myb, myc, fyn, and the translocation gene bcr/abl encode products that remain in the nucleus and are involved in transcription and cell cycle events. Gene products of oncogenes such as ras, src and P53 are generally found in the cytoplasm. Membrane bound products of oncogenes include neu, trk and EGRF. While protein products of these genes are often found in normal cells, they exist at greater levels in cancer cells. Thus, cancer cells can be expected to be more likely to have these proteins bound to Class I MHC molecules at the cell surface. Accordingly, CTLs which specifically recognize the target protein/MHC I complex will be more effective against cancer cells.

In addition to oncogene products as target antigens, variable regions of antibodies made by B cell lymphomas and variable regions of T cell receptors of T cell lymphomas can also be used as target antigens. These antigens are discussed and described in greater detail below in the section referring to autoimmune disease. However, it is contemplated that similar vaccination strategies can be used for treating and preventing these types of cancer.



Additionally, other tumor-associated proteins can be used as target proteins. Such proteins are generally those which are found at higher levels in tumor cells. Examples include the protein recognized by monoclonal antibody 17-1A and folate binding proteins.

While the present invention may be used to immunize an individual against one or more of several forms of cancer, the present invention is particularly useful to immunize an individual who is predisposed to develop a particular cancer or who has had cancer and is therefore susceptible to a relapse.

Developments in genetics and technology as well as epidemiology allow for the determination of probability and risk assessment for the development of cancer in individual. Using genetic screening and/or family health histories, it is possible to predict the probability a particular individual has for developing any one of several types of cancer. Those individuals identified as being predisposed to developing a particular form of cancer can, by using the methods of the present invention, take prophylactic steps towards reducing the risk of cancer. According to the present invention, high-risk individuals can be immunized against the form of cancer that they have a predisposition to develop.

Similarly, those individuals who have already developed cancer and who have been treated to remove the cancer or are otherwise in remission are particularly susceptible to relapse and recurrence. As part of a treatment regimen, such individuals can be immunized against the cancer that they have been diagnosed as having had in order to combat a recurrence. Thus, once it is known that an individual has had a type of cancer and is at risk of a relapse, they can be immunized in order to prepare their immune system to combat any future appearance of the cancer.

The present invention provides a method of treating individuals suffering from hyperproliferative diseases. In such methods, the introduction of genetic constructs serves as an immunotherapeutic, directing and promoting the immune system of the individual to combat hyperproliferative cells that produce the target protein.

The present invention provides a method of treating individuals suffering from autoimmune diseases and disorders by conferring a broad based protective immune response against targets that are associated with autoimmunity including cell receptors and cells which produce "self"-directed antibodies.

T cell mediated autoimmune diseases include Rheumatoid arthritis (RA), multiple sclerosis (MS), Sjogren's syndrome, sarcoidosis, insulin dependent diabetes mellitus (IDDM), autoimmune thyroiditis, reactive arthritis, ankylosing spondylitis, scleroderma, polymyositis, dermatomyositis, psoriasis, vasculitis, Wegener's granulomatosis, Crohn's disease and ulcerative colitis. Each of these diseases is characterized by T cell receptors that bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of the T cells would elicit an immune response including CTLs to eliminate those T cells.

In RA, several specific variable regions of T cell receptors (TCRs) which are involved in the disease have been characterized. These TCRs include V $\beta$ -3, V $\beta$ -14, V $\beta$ -17 and V $\alpha$ -17. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in RA. See: Howell, M.D., et al., 1991 *Proc. Natl. Acad. Sci. USA* 88:10921-10925; Paliard, X., et al., 1991 *Science* 253:325-329; Williams, W. V., et al., 1992 *J. Clin. Invest.*

90:326-333; each of which is incorporated herein by reference.

In MS, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include V $\beta$ -7 and V $\alpha$ -10. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in MS. See: Wucherpfennig, K. W., et al., 1990 *Science* 248:1016-1019; Oksenberg, J. R., et al., 1990 *Nature* 345:344-346; each of which is incorporated herein by reference.

In scleroderma, several specific variable regions of TCRs which are involved in the disease have been characterized. These TCRs include V $\beta$ -6, V $\beta$ -8, V $\beta$ -14 and V $\alpha$ -16, V $\alpha$ -3C, V $\alpha$ -7, V $\alpha$ -14, V $\alpha$ -15, V $\alpha$ -16, V $\alpha$ -28 and V $\alpha$ -12. Thus, vaccination with a DNA construct that encodes at least one of these proteins will elicit an immune response that will target T cells involved in scleroderma.

In order to treat patients suffering from a T cell mediated autoimmune disease, particularly those for which the variable region of the TCR has yet to be characterized, a synovial biopsy can be performed. Samples of the T cells present can be taken and the variable region of those TCRs identified using standard techniques. Genetic vaccines can be prepared using this information.

B cell mediated autoimmune diseases include Lupus (SLE), Grave's disease, myasthenia gravis, autoimmune hemolytic anemia, autoimmune thrombocytopenia, asthma, cryoglobulinemia, primary biliary sclerosis and pernicious anemia. Each of these diseases is characterized by antibodies which bind to endogenous antigens and initiate the inflammatory cascade associated with autoimmune diseases. Vaccination against the variable region of antibodies would elicit an immune response including CTLs to eliminate those B cells that produce the antibody.

In order to treat patients suffering from a B cell mediated autoimmune disease, the variable region of the antibodies involved in the autoimmune activity must be identified. A biopsy can be performed and samples of the antibodies present at a site of inflammation can be taken. The variable region of those antibodies can be identified using standard techniques. Genetic vaccines can be prepared using this information.

In the case of SLE, one antigen is believed to be DNA. Thus, in patients to be immunized against SLE, their sera can be screened for anti-DNA antibodies and a vaccine can be prepared which includes DNA constructs that encode the variable region of such anti-DNA antibodies found in the sera.

Common structural features among the variable regions of both TCRs and antibodies are well known. The DNA sequence encoding a particular TCR or antibody can generally be found following well known methods such as those described in Kabat, et al. 1987 *Sequence of Proteins of Immunological Interest* U.S. Department of Health and Human Services, Bethesda Md., which is incorporated herein by reference. In addition, a general method for cloning functional variable regions from antibodies can be found in Chaudhary, V. K., et al., 1990 *Proc. Natl. Acad. Sci. USA* 87:1066, which is incorporated herein by reference.

According to the invention, the genetic vaccine may be administered directly into the individual to be immunized or ex vivo into removed cells of the individual which are reimplanted after administration. By either route, the genetic material is introduced into cells which are present in the body of the individual. Routes of administration include, but



are not limited to, intramuscular, intraperitoneal, intradermal, subcutaneous, intravenous, intraarterially, intraocularly and oral as well as transdermally or by inhalation or suppository. Preferred routes of administration include intramuscular, intraperitoneal, intradermal and subcutaneous injection. Genetic constructs may be administered by means including, but not limited to, traditional syringes, needleless injection devices, or "microprojectile bombardment gene guns". Alternatively, the genetic vaccine may be introduced by various means into cells that are removed from the individual. Such means include, for example, *ex vivo* transfection, electroporation, microinjection and microprojectile bombardment. After the genetic construct is taken up by the cells, they are reimplanted into the individual. It is contemplated that otherwise non-immunogenic cells that have genetic constructs incorporated therein can be implanted into the individual even if the vaccinated cells were originally taken from another individual.

The genetic vaccines according to the present invention comprise about 1 nanogram to about 1000 micrograms of DNA. In some preferred embodiments, the vaccines contain about 10 nanograms to about 800 micrograms of DNA. In some preferred embodiments, the vaccines contain about 0.1 to about 500 micrograms of DNA. In some preferred embodiments, the vaccines contain about 1 to about 350 micrograms of DNA. In some preferred embodiments, the vaccines contain about 25 to about 250 micrograms of DNA. In some preferred embodiments, the vaccines contain about 100 micrograms DNA.

The genetic vaccines according to the present invention are formulated according to the mode of administration to be used. One having ordinary skill in the art can readily formulate a genetic vaccine that comprises a genetic construct. In cases where intramuscular injection is the chosen mode of administration, an isotonic formulation is preferably used. Generally, additives for isotonicity can include sodium chloride, dextrose, mannitol, sorbitol and lactose. In some cases, isotonic solutions such as phosphate buffered saline are preferred. Stabilizers include gelatin and albumin. In some embodiments, a vaso-constriction agent is added to the formulation. The pharmaceutical preparations according to the present invention are provided sterile and pyrogen free.

Genetic constructs may optionally be formulated with one or more response enhancing agents such as: compounds which enhance transfection, i.e. transfecting agents; compounds which stimulate cell division, i.e. replication agents; compounds which stimulate immune cell migration to the site of administration, i.e. inflammatory agents; compounds which enhance an immune response, i.e. adjuvants or compounds having two or more of these activities.

As used herein, the term "transfection agent" is meant to refer to an agent that promotes and facilitates the uptake of genetic material by the cells. According to the present invention, cells are contacted with one or more transfection agents prior to, simultaneously with or subsequent to administration of the genetic construct.

As used herein, the term "replicating agent" is meant to refer to an agent that stimulates cell division and replication. According to the present invention, cells are contacted with one or more replicating agents prior to simultaneously with, or subsequent to administration of the genetic construct.

As used herein, the term "inflammatory agent" is meant to refer to an agent that induces migration and chemotaxis of cells involved in an immune response to the site in an individual where it is administered. According to the present

invention, cells are contacted with one or more inflammatory agents prior to, simultaneously with, or subsequent to administration of the genetic construct. An inflammatory agent can be an irritant which disrupts or damages tissue. Thus, in addition to the cells that are normally present at the site of administration, the migrating immune cells can come into contact with and take up the administered genetic construct.

As used herein, the term "cell stimulating agent" refers to a compound that is both a transfection agent in that it facilitates DNA and RNA uptake by cells and a replicating agent in that it stimulates cell division and replication. As used herein, the terms "cell stimulating agent" or "cell proliferative agent" are used interchangeably and refer to compounds which are transfecting agents and replicating agents. Cell stimulating agents facilitate DNA and RNA uptake, and stimulate cell division.

In some embodiments, the transfecting agent used is preferably a cell stimulating agent. In some embodiments, a transfecting agent is used which is also an inflammatory agent. In some embodiments, a transfecting agent is used which is also an adjuvant. In some embodiments, a transfecting agent is used which is also an inflammatory agent and an adjuvant. In some embodiments, a cell stimulating agent is used which is also an inflammatory agent. In some embodiments, a cell stimulating agent is used which is also an adjuvant. In some embodiments, a cell stimulating agent is used which is also an inflammatory agent and an adjuvant. In some embodiments, a replicating agent is used which is also an inflammatory agent. In some embodiments, a replicating agent is used which is also an adjuvant. In some embodiments, a replicating agent is used which is also an inflammatory agent and an adjuvant. In some embodiments, an inflammatory agent is used which is also an adjuvant.

In a preferred embodiment, bupivacaine, a well known and commercially available pharmaceutical compound, is administered prior to, simultaneously with or subsequent to the genetic construct. Bupivacaine and the genetic construct may be formulated in the same composition. Bupivacaine is particularly useful as a cell stimulating agent in view of its many properties and activities when administered to tissue. Bupivacaine promotes and facilitates the uptake of genetic material by the cell. As such, it is a transfecting agent. Administration of genetic constructs in conjunction with bupivacaine facilitates entry of the genetic constructs into cells. Bupivacaine is believed to disrupt or otherwise render the cell membrane more permeable. Cell division and replication is stimulated by bupivacaine. Accordingly, bupivacaine acts as a replicating agent. Administration of bupivacaine also irritates and damages the tissue. As such, it acts as an inflammatory agent which elicits migration and chemotaxis of immune cells to the site of administration. In addition to the cells normally present at the site of administration, the cells of the immune system which migrate to the site in response to the inflammatory agent can come into contact with the administered genetic material and the bupivacaine. Bupivacaine, acting as a transfection agent, is available to promote uptake of genetic material by such cells of the immune system as well.

Bupivacaine is related chemically and pharmacologically to the aminoacyl local anesthetics. It is a homologue of mepivacaine and related to lidocaine. Bupivacaine renders muscle tissue voltage sensitive to sodium challenge and effects ion concentration within the cells. A complete description of bupivacaine's pharmacological activities can be found in Ritchie, J. M. and N. M. Greene, *The Pharmacological Basis of Therapeutics*, Eds.: Gilman, A. G. et al,

8th Edition, Chapter 15:3111, which is incorporated herein by reference. Bupivacaine and compounds that display a functional similarity to bupivacaine are preferred in the method of the present invention.

Bupivacaine-HCl is chemically designated as 2-piperidinecarboxamide, 1-butyl-N-(2,6-dimethylphenyl)monohydrochloride, monohydrate and is widely available commercially for pharmaceutical uses from many sources including Astra Pharmaceutical Products Inc. (Westboro, Mass.) and Sanofi Winthrop Pharmaceuticals (New York, N.Y.). Eastman Kodak (Rochester, N.Y.). Bupivacaine is commercially formulated with and without methylparaben and with or without epinephrine. Any such formulation may be used. It is commercially available for pharmaceutical use in concentration of 0.25%, 0.5% and 0.75% which may be used on the invention. Alternative concentrations which elicit desirable effects may be prepared if desired. According to the present invention, about 250 µg to about 10 mg of bupivacaine is administered. In some embodiments, about 250 µg to about 7.5 mg is administered. In some embodiments, about 0.50 mg to about 5.0 mg is administered. In some embodiments, about 1.0 mg to about 3.0 mg is administered. In some embodiments about 5.0 mg is administered. For example, in some embodiments about 50 µl to about 2 ml, preferably 50 µl to about 1500 µl and more preferably about 1 ml of 0.5% bupivacaine-HCl and 0.1% methylparaben in an isotonic pharmaceutical carrier is administered at the same site as the vaccine before, simultaneously with or after the vaccine is administered. Similarly, in some embodiments, about 50 µl to about 2 ml, preferably 50 µl to about 1500 µl and more preferably about 1 ml of 0.5% bupivacaine-HCl in an isotonic pharmaceutical carrier is administered at the same site as the vaccine before, simultaneously with or after the vaccine is administered. Bupivacaine and any other similarly acting compounds, particularly those of the related family of local anesthetics may be administered at concentrations which provide the desired facilitation of uptake of genetic constructs by cells.

In some embodiments of the invention, the individual is first subject to bupivacaine injection prior to genetic vaccination by intramuscular injection. That is, up to, for example, up to a about a week to ten days prior to vaccination, the individual is first injected with bupivacaine. In some embodiments, prior to vaccination, the individual is injected with bupivacaine about 1 to 5 days before administration of the genetic construct. In some embodiments, prior to vaccination, the individual is injected with bupivacaine about 24 hrs before administration of the genetic construct. Alternatively, bupivacaine can be injected simultaneously, minutes before or after vaccination. Accordingly, bupivacaine and the genetic construct may be combined and injected simultaneously as a mixture. In some embodiments, the bupivacaine is administered after administration of the genetic construct. For example, up to about a week to ten days after administration of the genetic construct, the individual is injected with bupivacaine. In some embodiments, the individual is injected with bupivacaine about 24 hrs after vaccination. In some embodiments, the individual is injected with bupivacaine about 1 to 5 days after vaccination. In some embodiments, the individual is administered bupivacaine up to about a week to ten days after vaccination.

In addition to bupivacaine, mepivacaine, lidocaine, procaine, carbocaine and methyl bupivacaine, other similarly acting compounds may be used as response enhancing agents. Such agents acts a cell stimulating agents which promote the uptake of genetic constructs into the cell and stimulate cell replication as well as initiate an inflammatory response at the site of administration.

Other contemplated response enhancing agents which may function transfecting agents and/or replicating agents and/or inflammatory agents and which may be co-administered with bupivacaine and similar acting compounds include lectins, growth factors, cytokines and lymphokines such as α-interferon, gamma-interferon, platelet derived growth factor (PDGF), gCSF, gMCSF, TNF, epidermal growth factor (EGF), IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12 as well as collagenase, fibroblast growth factor, estrogen, dexamethasone, saponins, surface active agents such as immune-stimulating complexes (ISCOMS), Freund's incomplete adjuvant, LPS analog including monophosphoryl Lipid A (MPL), muramyl peptides, quinone analogs and vesicles such as squalene and squalane, hyaluronic acid and hyaluronidase may also be used administered in conjunction with the genetic construct. In some embodiments, combinations of these agents are administered in conjunction with bupivacaine and the genetic construct. For example, bupivacaine and either hyaluronic acid or hyaluronidase are co-administered with a genetic construct.

The genetic construct may be combined with collagen as an emulsion and delivered parenterally. The collagen emulsion provides a means for sustained release of DNA. 50 µl to 2 ml of collagen are used. About 100 µg DNA are combined with 1 ml of collagen in a preferred embodiment using this formulation.

In some embodiments of the invention, the genetic construct is injected with a needleless injection device. The needleless injection devices are particularly useful for simultaneous administration of the material intramuscularly, intradermally and subcutaneously.

In some embodiments of the invention, the genetic construct is administered with a response enhancing agent by means of a microprojectile particle bombardment procedure as taught by Sanford et al. in U.S. Pat. No. 4,945,050 issued Jul. 31, 1990, which is incorporated herein by reference.

In some embodiments of the invention, the genetic construct is administered as part of a liposome complex with a response enhancing agent.

In some embodiments of the invention, the individual is subject to a single vaccination to produce a full, broad immune response. In some embodiments of the invention, the individual is subject to a series of vaccinations to produce a full, broad immune response. According to some embodiments of the invention, at least two and preferably four to five injections are given over a period of time. The period of time between injections may include from 24 hours apart to two weeks or longer between injections, preferably one week apart. Alternatively, at least two and up to four separate injections are given simultaneously at different sites.

In some embodiments of the invention, a complete vaccination includes injection of a single inoculant which contains a genetic construct including sequences encoding one or more targeted epitopes.

In some embodiments of the invention, a complete vaccination includes injection of two or more different inoculants into different sites. For example, in an HIV vaccine according to the invention, the vaccine comprises two inoculants in which each one comprises genetic material encoding different viral proteins. This method of vaccination allows the introduction of as much as a complete set of viral genes into the individual without the risk of assembling an infectious viral particle. Thus, an immune response against most or all of the virus can be invoked in the vaccinated individual. Injection of each inoculant is performed at different

sites, preferably at a distance to ensure no cells receive both genetic constructs. As a further safety precaution, some genes may be deleted or altered to further prevent the capability of infectious viral assembly. As used herein, the term "pharmaceutical kit" is meant to collectively refer to multiple inoculant used in the present invention. Such kits include separate containers containing different inoculants and/or cell stimulating agents. It is intended that these kits be provided to include a set of inoculants used in an immunizing method.

While the disclosure herein primarily relates to uses of the methods of the present invention to immunize humans, the methods of the present invention can be applied to veterinary medical uses too. It is within the scope of the present invention to provide methods of immunizing non-human as well as human individuals against pathogens and protein specific disorders and diseases. Accordingly, the present invention relates to genetic immunization of mammals, birds and fish. The methods of the present invention can be particularly useful for mammalian species including human, bovine, ovine, porcine, equine, canine and feline species.

While this disclosure generally discusses immunization in the context of prophylactic methods of protection, the term "immunizing" is meant to refer to both prophylactic and therapeutic methods. Thus, a method of immunizing includes both methods of protecting an individual from pathogen challenge or occurrence or proliferation of specific cells as well as methods of treating an individual suffering from pathogen infection, hyperproliferative disease or autoimmune disease. Accordingly, the present invention may be used as a vaccine for prophylactic protection or in a therapeutic manner; that is, as immunotherapeutic methods and preparations.

Other aspects of the invention include the use of bupivacaine and related, similarly acting cell stimulating agents in methods of introducing therapeutic genes into cells of an individual. Thus, one aspect of the present invention relates gene therapy; that is, to methods of introducing nucleic acid molecules that encode therapeutic proteins into the cells of an individual. The administration protocols and genetic constructs useful in gene therapy applications are the same as those described above for genetic immunization except the genetic constructs include nucleotide sequences that encode proteins whose presence in the individual will eliminate a deficiency in the individual and/or whose presence will provide a therapeutic effect on the individual.

The Examples set out below include representative examples of aspects of the present invention. The Examples are not meant to limit the scope of the invention but rather serve exemplary purposes. In addition, various aspects of the invention can be summarized by the following description. However, this description is not meant to limit the scope of the invention but rather to highlight various aspects of the invention. One having ordinary skill in the art can readily appreciate additional aspects and embodiments of the invention.

## EXAMPLES

### Example 1

According to the present invention, an effective vaccine has been produced which can invoke a protective immune response against HIV infected cells as well as cell free virus. As the awareness of AIDS and HIV infection has grown, repeated attempts and vast expenditures of resources and

efforts have been made to produce an HIV vaccine. Despite enormous efforts, little progress has been made thus far and the long felt need for an HIV vaccine has gone unabated.

The present invention provides an HIV vaccine using direct genetic immunization. Genetic constructs are provided which, when delivered into the cells of an individual, are expressed to produce HIV proteins. According to some embodiments, the production of all viral structural proteins in the cells of the individual elicit a protective immune response which protects against HIV infection. The HIV vaccine of the present invention may be used to immunize uninfected individuals from HIV infection or serve as an immunotherapeutic for those individuals already infected. The HIV vaccine of the present invention invokes an immune response including CTLs which recognize and attack HIV infected cells and recognize the widest contingent of HIV protein. Thus, uninfected individuals are protected from HIV infection.

In some embodiments, the present invention relates to a method of immunizing an individual against HIV by administering two inoculants. These two inoculants comprise at least two and preferably more than two, a plurality or all of the genes of the HIV virus. However, the inoculants are not delivered together. Accordingly, an inoculated cell will not be administered a complete complement of genes. The vaccinated individual will receive at least two different and preferably more than two, more preferably a plurality or all of the viral genes. Immune responses can then be directed at the total complement of HIV protein target.

This strategy serves two purposes. First, it is unknown which target protein is most effective as an immunizing antigen to protect an individual against infection. Thus, immunizing with two or more provides a greater probability that the vaccinated individual will be provided with sufficient immunogenic target proteins for eliciting a protective immune response. Secondly, HIV proteins are known to undergo structural changes due to mutation. By providing multiple antigenic targets, the probability that a viral particle will escape detection by the immune response is reduced despite structural changes in one or more viral proteins. Accordingly, it is desirable to vaccinate an individual with multiple and preferably a nearly complete or complete complement of genes encoding viral proteins.

If a single cell is provided with a complete complement of viral genes, it is possible that a complete infectious virus can be assembled within the cell. Accordingly, a genetic construct according to the present invention is not provided with such a full complement of genes. Furthermore, two or more inoculants, each having an incomplete set of genes and combined having up to a full complement of viral genes, are administered to different cells, preferably at a distant site from each other to ensure that no vaccinated cell will inadvertently be exposed to a full set of genes. For example, a portion of the HIV genome may be inserted into a first construct and the remaining portion of the HIV genome is inserted in a second construct. The first construct is administered to an individual as a genetic vaccine in the muscle tissue of one arm while the second construct is administered to an individual as a genetic vaccine in the muscle tissue of the individual's other arm. The individual may be exposed to a full set of viral genes; thus essentially vaccinating against the whole virus but with no risk that an infectious viral particle will be assembled.

As an additional safety precaution, even when genetic material is delivered by two or more inoculants at distant parts of the individual's body, one or more essential genes

can be deleted or intentionally altered to further ensure that an infectious viral particle cannot be formed. In such embodiments, the individual is not administered a complete functional set of viral genes.

A further safety precaution provides non-overlapping portions of the viral genome on the separate genetic constructs that make up the separate inoculants respectively. Accordingly, recombination between the two genetic constructs is prevented.

In some embodiments of the present invention, a full complement of structural genes are provided. The structural genes of HIV consist of gag, pol and env. These three genes are provided on two different DNA or RNA constructs. Accordingly, in one preferred embodiment, gag and pol are on one DNA or RNA construct and env is on another. In another preferred embodiment, gag is on one DNA or RNA construct and pol and env is on the other. In another preferred embodiment, gag and env are on one DNA or RNA construct and pol is on the other. Optionally, in any of these combinations, HIV regulatory genes may also be present. The HIV regulatory genes are: vpr, vif, vpu, nef, tat and rev.

The DNA construct in a preferred embodiment consists of a promoter, an enhancer and a polyadenylation signal. The promoter may be selected from the group consisting of: HIV LTR, human Actin, human Myosin, CMV, RSV, Moloney, MMTV, human Hemoglobin, human muscle creatine and EBV. The enhancer may be selected from the group consisting of: human Actin, human Myosin, CMV, RSV, human Hemoglobin, human muscle creatine and EBV. The polyadenylation signal may be selected from the group consisting of: LTR polyadenylation signal and SV40 polyadenylation signal, particularly the SV40 minor polyadenylation signal among others.

In some embodiments, the two inoculant vaccine is administered intramuscularly at spatially segregated tissue of the individual, preferably in different appendages, such as for example in the right and left arms. Each inoculant of the present invention may contain from about 0.1 to about 1000 micrograms of DNA. Preferably, each inoculant contains about 1 to about 500 micrograms of DNA. More preferably, each inoculant contains about 25 to about 250 micrograms of DNA. Most preferably, each inoculant contains about 100 micrograms DNA.

The inoculant in a preferred embodiment is in a sterile isotonic carrier, preferably phosphate buffered saline or saline solution.

In some embodiments, prior to vaccine administration, the tissue to be vaccinated is injected with a cell proliferating agent, preferably bupivacaine. Bupivacaine injections may be performed up to about 24 hours prior to vaccination. It is contemplated that bupivacaine injection will occur immediately before vaccination. About 50  $\mu$ l to about 2 ml of 0.5% bupivacaine-HCl and 0.1% methylparaben in isotonic NaCl is administered to the site where the vaccine is to be administered, preferably, 50  $\mu$ l to about 1500  $\mu$ l, more preferably about 1 ml.

In other embodiments, a cell proliferating agent, preferably bupivacaine is included in the formulation together with the genetic construct. About 50  $\mu$ l to about 2 ml of 0.5% bupivacaine-HCl and 0.1% methylparaben in isotonic NaCl is administered to the site where the vaccine is to be administered, preferably, 50  $\mu$ l to about 1500  $\mu$ l, more preferably about 1 ml.

Accordingly, some embodiments comprise a two inoculant vaccine: one inoculant comprising a DNA or RNA construct having two HIV structural genes, the other inocu-

lant comprising a DNA or RNA construct having the third, remaining HIV structural gene such that the combined inoculants contain a full complement of HIV structural genes. The structural genes on each DNA construct are operably linked to a promoter, an enhancer and a polyadenylation signal. The same or different regulatory elements may control expression of the viral genes. When vaccinating an individual, the two inoculants are administered intramuscularly to different sites, preferably on different arms. In some embodiments of the invention, bupivacaine is first administered at the site where inoculant is to be administered. In some embodiments of the invention, bupivacaine is included in the formulations together with the genetic constructs.

In some embodiments, the vaccination procedure is repeated at least once and preferably two or three times. Each vaccination procedure is performed from 24 hours to two months apart.

In some embodiments, the vaccine is administered using a needleless injection device. In some embodiments, the vaccine is administered hypodermically using a needleless injection device thus providing intramuscular, intradermal, subcutaneous administration simultaneously while also administering the material interstitially.

Preferred genetic constructs include the following. Plasmids and Cloning Strategies:

Two plasmids were constructed: one which contains HIV gag/pol and the other which contains HIV env.

The HIV-1 genomic clone pNL43 was obtained through the NIH AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, NIAID, NIH, from Dr. Malcolm Martin, and can be used as the starting material for HIV-1 viral genes for genetic constructs. Alternatively, any HIV molecular clone of infected cell can, through use of the polymerase chain technology, be modified sufficiently for construction including the HXB2 clone the MN clone as well as the SF or BAL-1 clone. The pNL43 clone is a construct that consists of HIV-1 proviral DNA plus 3 kb of host sequence from the site of integration cloned into pUCIS.

Construction of pNL-puro-env plasmid:

This plasmid was constructed for expression of gag pol. The StuI site within the non-HIV 5' flanking human DNA of pNL43 was destroyed by partial digestion with StuI followed by digestion of the free ends with *E. coli* polymerase I. The linear plasmid was filled and then self ligated, leaving a unique StuI site within the HIV genome. This plasmid, pNLdStu, was then digested with the blunting enzymes StuI and BsaBI which eliminated a large section of the coding sequence for gp120. The SV40 promoter and puromycin resistance coding region (puromycin acetyl transferase (PAC)) were isolated from pBABE-puro (Morgenstern and Land, 1990 *Nucl. Acids Res.* 18(12):3587-3596, which is incorporated herein by reference, kindly provided by Dr. Hartmut Land of the Imperial Cancer Research Fund) using EcoRI and ClaI. This fragment was blunted, then cloned into the StuI/BsaBI-digested pNLdStu. A clone was selected with the SV40-puro fragment in the correct orientation so that the 3' LTR of HIV could provide poly A functions for the PAC message. This plasmid was designated pNLpuro. Cloning strategy for deletion of vpr regulatory gene from the HIV gag pol vector.

A region from just upstream of the unique PflMI site to just after the vif termination codon was amplified via PCR using primers that introduced a non-conservative amino acid change (glu→val) at aminoacid 22 of vpr, a stop codon in the

vpr reading frame immediately after amino acid 22, and an EcoRI site immediately following the new stop codon. This PCR fragment was substituted for the PflMI-EcoR I fragment of pNLpuro or pNL43. This substitution resulted in the deletion of 122 nucleotides of the open reading frame of vpr, thus eliminating the possibility of reversion that a point mutation strategy entails. The resulting plasmids, pNLpuroAvpr, encode the first 21 natural amino acids of vpr plus a valine plus all other remaining HIV-1 genes and splice junctions in their native form. Such deletion strategy would also be applicable to nef, vif, and vpu and allow for structural gene expression but protect from the generation of a live recombinant virus.

#### Plasmid construction for envelope expression:

The DNA segment encoding the envelope gene of HIV-1 HXB2 was cloned by the polymerase chain reaction (PCR) amplification technique utilizing the lambda cloned DNA obtained from the AIDS Research and Reference Reagent Program. The sequences of the 5' and 3' primers are 5'-AG-GCGTCTCGAGACAGAGGAGACCAAGAAATG-3' (SEQ ID NO:1) with incorporation of XhoI site and 5'-TTTCCCTCTAGATAAGCCATCCAATCACAC-3' (SEQ ID NO: 2) with incorporation of XbaI site, respectively, which encompass gp160, tat and rev coding region. Gene specific amplification was performed using Taq DNA polymerase according to the manufacturer's instructions (Perkin-Elmer Cetus Corp.). The PCR reaction products were treated with 0.5 ug/ml proteinase K at 37° C. for thirty minutes followed by a phenol/chloroform extraction and ethanol precipitation. Recovered DNA was then digested with XhoI and XbaI for two hours at 37° C. and subjected to agarose gel electrophoresis. The isolated and purified XhoI-XbaI PCR fragment was cloned into Bluescript plasmid (Stratagene Inc., La Jolla, Calif.) and then subcloned into the eukaryotic expression vector pMAMneoBlue (Clontech Laboratories, Inc., Palo Alto, Calif.). The resulting construct was designated as pM160. The plasmid DNA was purified with CsCl gradient ultracentrifugation. An alternative envelope expression plasmid construction called HIV-1 env-rev plasmid:

The region encoding the two exons of rev and the vpu and envelope open reading frames of HIV-1 HXB2 was amplified via PCR and cloned into the expression vector pCND4/neo (Invitrogen). This plasmid drives envelope production through the CMV promoter.

#### Production and Purification:

The plasmid in *E. coli* (DH5 alpha) is grown up as follows: An LB plus ampicillin agar plate is streaked with the desired plasmid culture from frozen stock. The plate is incubated overnight (14-15 hours) at 37° C. A single colony is taken from the plate and inoculated into 15 ml of LB medium with a peptone preparation and 50 µg/ml ampicillin. This culture is grown at 37° C. while being shaken (ca. 175 rpm) for 8-10 hours. OD<sub>600</sub> readings should be at least 1.0. 1 liter of LB medium with peptone and 50 µg/ml ampicillin is inoculated with 1.0 OD of culture. These 1-2 liter cultures are grown overnight at 37° C. while being shaken (175 rpm).

Plasmid grown in *E. coli* (strain DH5 alpha) are harvested and purified by the following methods. General procedures for the lysis of cells and purification of plasmid can be found in "Molecular Cloning: A Laboratory Manual", 2nd Edition, J. Sambrook, E. F. Fritsch, and T. Maniatis, Cold Spring Harbor Press, 1989. The cells are concentrated and washed with glucose-tris-EDTA pH 8.0 buffer. The concentrated cells are lysed by treatment with lysozyme and briefly treated with 0.2N KOH, the pH is then adjusted 5.5 with potassium acetate/acetic acid buffer. Insoluble material is

removed by centrifugation. To the supernatant is added 2-propanol to precipitate the plasmid. The plasmid is redissolved in tris-EDTA buffer and further purified by phenol/chloroform extraction and an additional precipitation with 2-propanol.

Endotoxin can optionally be removed by a variety of methods including the following: specific adsorption by immobilized materials such as polymyxin ("Endotoxin removed from hemoglobin solution using polymyxin B-immobilized fibre (PMX-F) . . .", Tani et al., *Biomater Artif Cells Immobilization Biotechnol.* 20(2-4):457-62 (1992); "Efficient endotoxin removal with a new sanitizable affinity column: Affi-Prep Polymyxin", Issekutz *J. Immunol Methods* 61(3):275-81 (1983)); anti-endotoxin monoclonal antibodies, such as 8A1 and HA-1A™ (Centocor, Malvern, Pa.; "Human Monoclonal Antibody HA-1A Binds to Endotoxin via an Epitope in the Lipid A Domain of Lipopolysaccharide" Bogard et al. *J. Immunol.* 150(10):4438-4449 (1993); Rietschel et al., *Infect. Immunity* page 3863 (1993)); positively charged depth filters ("Depyrogenation by endotoxin removal with positively charged depth filter cartridge", Hou et al., *J. Parenter Sci Technol.* 44(4):204-9 (July-August 1990)); poly(gamma-methyl L-glutamate) ("Removal of endotoxin from culture supernatant of Bortedella pertussis with aminated poly (gamma-methyl L-glutamate) spherical beads", Hirayama et al., *Chem. Pharm. Bull. (Tokyo)* 40(8):2106-9 (1992)); histidine ("Specific removal of endotoxin from protein solutions by immobilized histidine", Matsumae et al., *Biotechnol. Appl. Biochem.* 12(2):129-40 (1990)); hydrophobic interaction columns and membranes (e.g., "Removal of endotoxin from protein solutions by phase separation using Triton X-114", Aida et al., *J. Immunol Methods* 132(2):191-5 (1990); "Novel endotoxin adsorbing materials, polymyxin-sepharose and polyporous polyethylene membrane for removal of endotoxin from dialysis systems", Umeda et al., *Biomater Artif Cells Artif Organs* 18(4):491-7 (1990); "The effect of hydrophobic interaction on endotoxin adsorption by polymeric affinity matrix", Hou et al., *Biochem. Biophys. Acta* 1073(1):149-54 (1991); "Endotoxin removal from water using microporous polyethylene chopped fibres as a new adsorbent", Sawada et al., *J. Hyg. (London)* 97(1):103-14 (1986)); specific hydrophobic resins useful for removing endotoxin including hydrophobic polystyrene/divinylbenzene or divinylbenzene resins such as Brownlee Polypore Resin (Applied Biosystems, Palo Alto, Calif.); XUS 40323.00 (Dow Chemical, Midland, Mich.); HP20, CHP20P (Mitsubishi Kasei, U.S.); Hamilton PRP-1, PRP-infinity (Hamilton, Reno, Nev.); Jordi Reversed-Phase DVB, Jordi Gel DVB, Polymer Labs PLgel™ (Alltech, Deerfield, Ill.); Vydac PLx™ (Separations Group, Hesperia, Calif.); other endotoxin removing materials and methods include Detoxi-Gel™ Endotoxin Removing Gel (Pierce Chemical, Rockford, Ill.); Application Note 206, "Chromatographic removal of endotoxins and/or ethanol from albumin", (Pharmacia Biotech Inc, Piscataway, N.J.) See also generally, "Endotoxin Detection and Elimination in Biotechnology", Sharma, *Biotech. App. Biochem.* 8:5-22 (1986).

Preferred anti-endotoxin monoclonal antibodies bind to the conserved domains of endotoxin, preferably antibodies to lipid A, the most structurally conserved portion of the endotoxin molecule. Such anti-lipid A monoclonal antibodies include the high affinity murine IgG monoclonal antibody 8A1 and the human anti-lipid A IgM(k) monoclonal antibody HA-1A™. HA-1A™ was derived from a human B *E. coli* J5 vaccine. HA-1A™. HA-1A™ is reported to be broadly cross-reactive with a variety of bacterial endotoxins (lipopolysaccharides).

## Example 2

In experiments designed to compare the immunogenic response elicited by genetic vaccination and protein vaccination, animal models were designed using tumor cells that specifically express a foreign target protein. Three immune competent mouse models have been developed which express foreign antigens. Three clonal tumor cell lines which are derived from the Balb/c mouse strain are used. The cell lines are: 1) a lymphoid cell line which does not metastasize significantly to other tissues but forms large palpable tumors which appear to kill the animal within an 8–12 week period; 2) a murine melanoma cell line with some ability to metastasize, mostly to the lung, and in which, following inoculation with 1 million cells, results in the development in the mice of large palpable tumors which similarly kill the animal within 10–12 weeks; and 3) a murine lung adenocarcinoma cell line which metastasizes to multiple tissues and kills the animal within 12 or more weeks. Subclones have been selected which can display foreign antigens in an unrecognized form. When transfected tumors are implanted into a parent mouse strain, unlike the majority of similar murine tumor lines, the animals do not make a protective immune response to the foreign antigens displayed and the tumors are accepted. These tumors then kill the animal with the same phenotype in the same time frame as the original untransfected tumor. Using these models, the immune response elicited by genetic vaccination against an antigen can be measured.

It was observed that mice vaccinated with a genetic vaccine comprising a genetic construct that resulted in production of the target protein by the cells of the mouse elicited an immune response including a strong cytotoxic that completely eliminated tumors displaying the target protein but with no effect on tumors that did not. In mice inoculated with the target protein itself, the immune response elicited thereby was much less effective. The tumors were reduced in size but, due to an absence of a cytotoxic response, they were not eliminated. As controls, untransfected tumors were used in experiments comparing the immune response of animals vaccinated with the genetic vaccine, subunit vaccine and unvaccinated animals. These experiments clearly demonstrate that the genetic vaccine produced a broader, more effective immune response which was capable, by virtue of CTL's, of completely eliminating tumors. By contrast, immunization using intact target protein produced a more limited, less effective immune response.

## Example 3

In some embodiments of the invention, the infectious virus, HIV, which is responsible for AIDS is the pathogenic agent against which a genetic vaccine has been designed. The viral protein gp160, which is processed into gp120 and gp41, is the target protein against which a genetic vaccine is produced. The genetic vaccine contains a DNA construct that comprises a DNA sequence encoding gp160 operably linked regulatory elements. When administered to an individual, the DNA construct of the genetic vaccine is incorporated into the cells of the individual and gp160 is produced. The immune response that is elicited by the protein is broad based and includes the humoral and both arms of the cellular immune response. The broad biological response provides superior protection to that achieved when the protein itself is administered.

The following is a description of the use of genetic immunization for elicitation of an anti-human immunodeficiency

virus type 1 (HIV-1) immune response in mice by administering a DNA construct that contains a DNA sequence which encodes the HIV envelope glycoprotein gp160. The gp160 construct (pM160) expresses biologically active HIV-1 envelope proteins in vivo.

Mice were injected intramuscularly with pM160 and subsequently analyzed for anti-HIV immune responses. The antisera from animals immunized in this manner produce anti-HIV envelope glycoprotein immune responses as measured by enzyme linked immunosorbent assay (ELISA) and immunoprecipitation assays. The antisera neutralizes HIV-1 infection and inhibits HIV-1 induced syncytium formation.

The observed neutralization and anti-syncytial activity may be the result of reactivity of the elicited antibodies to functionally important regions of the HIV-1 envelope protein, such as the V3 loop of gp120, CD4 binding site and the N-terminal "immunodominant" region of gp41, among others.

The DNA construct (pM160) encoding the HIV-1/HXB2 (Fisher, A. G., et al., (1985) *Nature* 316:262–265) gp160 membrane bound glycoprotein under control of a RSV enhancer element with the MMTV LTR as a promoter (FIG. 1A) was tested to determine whether this membrane-bound protein, when expressed by endogenous cells, can generate an anti-pathogen immune responses. The construct was generated as follows. The DNA segment encoding the envelope gene of HIV-1 HXB2 was cloned by the polymerase chain reaction (PCR) technique amplification utilizing the lambda cloned DNA obtained from ch AIDS repository. The sequences of the 5' and 3' primers are 5'-AGGCGTCTCGAGACAGAGGAGAGCAA-GAAATG-3' (SEQ ID NO:11) with incorporation of XhoI site and 5'-TTTCCTCTAGATAAGCCATCCAATCAC-3' (SEQ ID NO:12) with incorporation of XbaI site, respectively, which encompass gp160, tat and rev coding region. Gene specific amplification was performed using Taq DNA polymerase according to manufacturer's instruction (Perkin-Elmer Cetus Corp.) The PCR reaction products were treated with 0.5 µg/ml proteinase K at 37° C. for thirty minutes followed by a phenol/chloroform extraction and ethanol precipitation (Crowe, J. S., et al., (1991) *Nucl. Acids Res.* 19:184). Recovered DNA was then digested with XhoI and XbaI for two hours at 37° C. and subjected to agarose gel electrophoresis. The isolated and purified XhoI-XbaI PCR fragment was cloned into Bluescript plasmid (Stratagene Inc., La Jolla, Calif.) and then subcloned into the eukaryotic expression vector pMAMneoBlue (Clontech, Inc.). The resulting construct was designated as pM160. The plasmid DNA was purified with CsCl purification (Sambrook, J. et al., (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.).

The pM160 construct, which contains a DNA sequence that encodes gp160, was transfected into a human rhabdomyosarcoma cell line, TE671 (Stratton, M. R., et al., (1989) *Carcinogenesis* 10:899–905), to evaluate its expression before introduction into living animals. Transfection of pM160 construct into TE671 cells was performed according to Wang, B., et al., (1992) *AIDS Human Retr.*, in press. Briefly, 2 µg of purified pM160 was added to 2x10<sup>6</sup> TE671 cells (Stratton, M. R., et al., (1989) *Carcinogenesis* 10:899–905) and subject to electroporation. Following electroporation, the cells were grown in fresh medium for forty eight hours prior to the addition of 500 µg/ml neomycin for selection. Individual cells expressing gp160 envelope protein were isolated by binding to M450 magnetic beads (Dynal) which was coated with mixture of monoclonal

anti-gp120 antibodies, namely ID6, AD3 and AC4 (Ugen, K. E. et al., (1992) *Generation of Monoclonal Antibodies Against the Amino Region of gp120 Which Elicits Antibody Dependent Cellular Cytotoxicity*, Cold Spring Harbor Laboratory, 1992). Clones were isolated by limiting dilution of the gp160 expressing cells. One of such clone was designated as the 3G7 cell line. Expression of gp120 and gp41 was determined by Western blot analysis of whole cell lysates of 3G7 cells versus vector-alone transfected TE671 cells, performed as previously described (Osther, K., et al., (1989) *Transplantation* 47:834-8; and Weiner, D. B., et al., (1989) *Vaccines*, Cold Spring Harbor Press, 115-120).

Typically, the mature HIV envelope glycoprotein gp160 is processed into gp120 and gp41 (Kowalski, M., et al., (1987) *Science* 237:1351-1355). The expression of HIV gp120 and gp41 by the pM160 transfected cell line 3G7 were observed in Western blot analysis with anti-gp160 specific serum (Osther, K., et al., (1991) *Hybridoma* 10:673-683) (FIG. 1B). Functional expression of gp160 by this cell line was further demonstrated by the ability of 3G7 but not TE671 cells to fuse with several CD4<sup>+</sup> T-cell cell lines.

In the genetic immunization procedure described herein, the quadriceps muscles of BALB/c mice were injected with 100  $\mu$ l of 0.5% bupivacaine-HCl and 0.1% methylparaben in isotonic NaCl using a 27-gauge needle to stimulate muscle cell regeneration and facilitate uptake of the genetic construct. Twenty-four hours later, the same injection sites were then injected with either 100  $\mu$ g of pM160 or with 100  $\mu$ g of pMAMneoBlue as a control plasmid (FIG. 1A). The mice were boosted by injecting the same amount of DNA construct three times at two week intervals in the same manner but without pre-treatment with bupivacaine-HCl.

For the recombinant gp160 immunization, BALB/C mice were initially immunized with 1 g of glycosylated recombinant (HIV-1/III<sub>B</sub>) gp160 (MicroGeneSys Inc.) in complete Freund's adjuvant followed by three boosters of 1  $\mu$ g of gp160 each in incomplete Freund's adjuvant at two week intervals. The production of antibody against HIV-1 gp160 was determined by testing the mouse sera for their ability to immunoprecipitate gp160. Immunoprecipitation was performed using  $1 \times 10^6$  cpm of <sup>125</sup>I labeled rgp160, mouse sera and protein-G agarose beads (GIBCO, Inc.) as previously described by Osther, K., et al., (1991) *Hybridoma* 10:673-683, which is incorporated herein by reference. The specific precipitations were analyzed by 10% SDS-PAGE. Lane 1 is 1  $\mu$ l of preimmune mouse serum reacted with the <sup>125</sup>I-gp160. Lane 2 is 1  $\mu$ l of mouse serum immunized from the pM160 immunized mice. Lane 3 is 1  $\mu$ l of 1:100 dilution of ID6 monoclonal anti-gp120 antibody (Ugen, K. E., et al., (1992) *Generation of Monoclonal Antibodies Against the Amino Region of gp120 Which Elicits Antibody Dependent Cellular Cytotoxicity*, Cold Spring Harbor Laboratory) as a positive control. The arrow indicates the specifically immunoprecipitated <sup>125</sup>I-gp160 envelope glycoprotein.

<sup>125</sup>I-labelled gp160 was specifically immunoprecipitated with antisera derived from the pM160-immunized animals (FIG. 2, lane 2) as well as the positive control anti-gp120 monoclonal antibody, ID6 (FIG. 2, lane 3). In contrast, the preimmune sera (FIG. 2, lane 1) only showed minimal activity in the same assay.

Eight of ten mice immunized with the pM160 construct were positive for reactivity against gp160 as determined by ELISA and the immune responses from the animal with the highest anti-gp160 titer was analyzed in detail. Four mice immunized with the control vector all showed a similar negative reactivity to gp160 in ELISA and one of these sera was used as the control for subsequent experiments.

It has been shown that HIV neutralizing antibodies are specifically targeted to several epitopes in gp120 and gp41, which include the V3 loop in gp120 (Goudsmit, J. et al., (1988) *AIDS* 2:157-164; and Putney, S. D., et al., (1989) *Development Of An HIV Subunit Vaccine*, Montreal), the CD4 binding site near the carboxy terminus of gp120 (Lasky, L. A., et al., (1987) *Cell* 50:975-985) as well as the immunodominant loop of gp41 just downstream of the N-terminal fusion region (Schrier, R. D., et al., (1988) *J. Virol.* 62:2531-2536).

To determine whether the anti-gp160 antibodies elicited in these mice are reactive to these important regions of the envelope glycoproteins, peptides for the BRU/V3 loop, peptides for the MN/V3 loop, peptides for the HXB2/gp41 N-terminus or peptides for HXB2/CD4 binding site were absorbed to microtiter plates and specific reactivities of the mouse antisera determined in ELISA assays. One  $\mu$ g/ml of gp160 or 10  $\mu$ g/ml of each peptide was coated to microtiter plates in 0.1M bicarbonate buffer (pH 9.5) overnight at 4° C., blocked with 2% bovine serum albumin in PBS, and reacted with goat anti-mouse IgG conjugated with HRPO (Fisher) for one hour at 37° C. and developed with TMB substrate (Sigma) for 10-30 minutes at room temperature in the dark. Results are reported in FIG. 3. Antisera were as follows: (+) is preimmune sera, (-x-) is the pMAMneoBlue vector immunized sera, (-O-) is the pM160 immunized sera, (-A-) is from mice immunized with the rgp160 protein. FIG. 3A shows results using a rgp160 protein coated plate. FIG. 3B shows results using a BRU/V3 loop peptides (CNTRKR-IRIQRGPGRAFVTIGK (SEQ ID NO:13)) coated plate. FIG. 3C shows results using a plate coated with MN/V loop peptides (YNKRKRIRIQRGPGRAFYTITKNIC (SEQ ID NO:14)) with the QR sequence from HIV-1/III<sub>B</sub> in bold-faced type. FIG. 3D shows the results using a HXB2/CD4 binding site peptides (CRIKQFINMWQEVGKAMYAP-PISGIRC (SEQ ID NO:15)) coated plate. FIG. 3E shows the results using a BRU/gp41 immunodominant region peptides (RLAVERYIKDQQLGIWGCSGKLC (SEQ ID NO:16)) coated plate.

For the recombinant gp160 immunization, BALB/C mice were initially immunized with 1  $\mu$ g of glycosylated recombinant (HIV-1/III<sub>B</sub>) gp160 (MicroGeneSys Inc.) in complete Freund's adjuvant followed by three boosters of 1  $\mu$ g of gp160 each in incomplete Freund's adjuvant at two week intervals.

FIG. 3 shows that antiserum from the pM160 construct immunized mouse has significantly higher reactivity to the BRU and MN/V3 loop peptides, the CD4 binding site peptide and the immunodominant gp41 peptide than the recombinant gp160 protein (rgp160) immunized serum. Interestingly, the antiserum from the rgp160 immunized mouse had much higher titer against the rgp160 than the pM160 immunized antiserum, but lower activity against the three specific neutralization epitopes of gp160 tested (FIG. 3a-d).

To determine whether the antisera generated by DNA immunization possessed antiviral activity, the ability of the antisera to neutralize HIV-1 infection was examined. Cell-free HIV-1/III<sub>B</sub> virus at 100 TCID<sub>50</sub> was incubated with serial dilutions of the antisera before being used to infect MT-2 target cells (Montefiori, D.C., (1988) *J. Clin. Microbio.* 26:231-235).

One hundred TCID<sub>50</sub> HIV-1/III<sub>B</sub> cell-free virus was pre-incubated with serial dilutions of antisera for one hour at 37° C. Following incubation the pretreated virus was then plated on the  $4 \times 10^4$  of target cell line, MT-2 for one hour at 37° C.,



following infection the MT-2 cells were washed three times and then incubated at 37° C. at 5% CO<sub>2</sub>. Fusion was evaluated three days later quantitatively by visually counting the number of syncytia per well in triplicate experiments under a phase contrast microscope.

The results are reported in FIG. 4. FIG. 4A shows the results using vector-immunized mouse sera compared with FIG. 4B which shows the results using pM160 immunized sera. Neutralization values ( $V_{50}/V_{50}$ ) versus the dilution factors (Nara, P., (1989) *Techniques In HIV Research* eds. Aldovini, A. & Walker, B. D., 77-86M Stockton Press) are illustrated in FIG. 4C. The control serum (-x) was from pMAMneoBlue vector immunized mice. The test sera (○) were from pM160 immunized mice.

Syncytia inhibition was performed as described by Oster, K., et al., (1991) *Hybridoma* 10:673-683. The H9/III<sub>B</sub> cell line was pre-incubated with serial dilutions (1:100, 1:200, and 1:400) of antisera were made in 96 well plates in a total volume of 50 µl for thirty minutes at 37° C. at 5% CO<sub>2</sub>. Fusion was evaluated three days later quantitatively by visually counting the number of syncytia per well under a phase contrast microscope. FIG. 4D is the target cells co-cultivated with HIV-1/III<sub>B</sub> cell line treated with preimmune serum. FIG. 4E is the same as FIG. 4D but treated with vector control immunized serum. FIG. 4F is the same as FIG. 4D but treated with rgp160 immunized serum. FIG. 4G is the same as FIG. 4D but treated with pM160 immunized serum. FIGS. 4D to 4G show that inhibition of syncytia was apparent at dilution at 1:200 in these assays. MT-2 cells were infected with cell-free HIV-1/III<sub>B</sub> which had been preincubated with vector-immunized antiserum readily formed syncytia (FIG. 4A). In comparison, preincubation with pM160 immunized mouse serum prevented syncytium formation (FIG. 4B). The neutralization kinetics were determined by  $V_{50}/V_{50}$  versus serial dilutions of antisera (Nara, P., (1989) *Techniques In HIV Research*, eds. Aldovini, A. & Walker, B. D., 77-86, M Stockton Press) (FIG. 4C). The serum from the pM160 immunized mouse had biologically active neutralizing activity at dilutions of up to 1:320 while control antisera did not show similar activity.

To determine if the antiserum from the pM160 immunized mouse could inhibit envelope-mediated virus spread through direct cell-to-cell fusion, syncytium inhibition assays were performed. Antiserum from the pM160 immunized mouse inhibits HIV-1 induced syncytium formation at 1:200 dilutions (FIG. 4G). In contrast, the preimmune sera (FIG. 4D), antisera from the rgp160 immunized mice (FIG. 4F) and antisera from the control vector-immunized animals (FIG. 4E) failed to inhibit syncytium formation at the same dilutions.

Observations from the neutralization (FIGS. 4A-C) and syncytium inhibition assays (FIGS. 4D-G) of these sera correlates with the observed ELISA reactivities (FIG. 3). The antiserum from the pM160 immunized mouse which showed a high level of binding to neutralizing epitopes likewise demonstrated high level anti-viral activities; conversely, sera with little binding to these epitopes including the antiserum from rgp160 immunized mice have low anti-viral activity.

Low level neutralizing activity has been observed by other groups when using rgp160 immunization (Lasky, L. A. et al., (1986) *Science* 233:209-212; and Berman P. W., et al., (1990) *Nature* 345:622-625. The reasons for the more effective generation of anti-viral activities by the genetic immunization than by recombinant protein immunization

are not clear. However, the differences in the generated immune responses may be due to the introduction of the gp160 gene directly into the mouse muscle cells and expression of this gene in vivo which may correctly process the products and lead to more effective processing of the target antigen.

HIV enters cells binding to the CD4 molecule found predominantly on human helper T-cells, macrophages, and possibly glial cells (Maddon, P. J., et al., (1986) *Cell* 47:333-348; Koenig, S., et al., (1986) *Science* 233:1089-1093; and Cheng-Mayer, C., et al., (1987) *Proc. Natl. Acad. Sci. USA* 84:3526-3530). Interruption of this binding has been shown to prevent HIV infection in vivo (Fisher, R. A., et al., (1988) *Nature* 331:76-78; and Sun, H. C., et al., 1989 *J. Virol.* 63:3579-85).

To test whether the antisera from pM160 immunized mice can inhibit gp120 binding to CD4-bearing T-cells, a direct inhibition assay monitored by fluorocytometry was employed (Chen, Y. H., et al., (1992) *AIDS* 6:533-539. It was observed that serum from the pM160 construct-immunized mouse was able to block the binding of gp120 to CD4-bearing T-cells: a 1:15 dilution of immune serum inhibited FITC-gp120 binding to CD4<sup>+</sup>SupT1 cells by 22%±2% in replicate experiments as evaluated by flow cytometry. This indicates that this region for HIV entry into target cells can also be functionally inhibited by this antiserum. These data are consistent with observed ELISA reactivity of the antiserum to the CD4 binding site peptides (FIG. 3c).

Immunoglobulin isotyping studies were performed by using a commercial murine monoclonal antibody isotyping kit (Sigma). Of the anti-gp160 specific antibodies elicited by pM160 immunization, 19% are IgG1, 51% are IgG2, 16% are IgG3, 10% are IgM and 5% are IgA. The predominance of IgG isotypes indicates that a secondary immune response has taken place, and further suggests that helper T-cells can be elicited by genetic immunization.

To determine whether immunization with the DNA construct can lead to the generation of anti-DNA antibodies in these experimental animals, pM160 and pMAMneoBlue DNAs were coated onto microtiter plates and specific binding was determined by ELISA using sera all immunized animals. No significant binding to plasmid DNA was observed. Thus, using genetic material for inoculation into muscle tissue appears unlikely to produce an anti-plasmid DNA response.

Introducing construct DNA into mouse muscle by needle injection may cause inconsistent results, as this technique does not provide a means to control DNA uptake by muscle cells. Injection of construct DNA alone (n=4) with bupivacaine pretreated animals (n=4) was compared. The immune responses observed in the two groups were dissimilar, with 25% and 75% animals responding in ELISA assays respectively. Increased efficiency may be achieved by use of a direct DNA delivery system such as particle bombardment (Klein, T. M. et al., (1992) *Biotechnology* 10:286-291).

Evidence of neutralization, syncytia inhibition, inhibition of CD4-gp120 binding, and specific binding to several important regions on the gp160 demonstrate that introduction of a DNA construct encoding HIV gp160 membrane-bound glycoprotein directly into muscle cells of living animals can elicit specific humoral responses, and generate biologically relevant anti-viral antibodies.

To test whether the vaccine is capable of eliciting a protective immune response, the animal model described above was used. Tumor cells were transfected with DNA



## 33

encoding p160, confirmed to express the protein and implanted into the animal. Controls included untransfected tumor lines.

Genetically immunized animals were vaccinated with plasmid pml60. Controls included unvaccinated animals, animals vaccinated with vector DNA only and animals administered the gp160 protein.

Results demonstrate that the immune response of genetically vaccinated mice was sufficient to completely eliminate the transfected tumors while having no effect on untransfected tumors. gp160 protein vaccination led to some reduction in tumor size in transfected tumors as compared to untransfected tumors but had no effect on mortality. Unvaccinated animals showed similar mortality for both transfected and untransfected tumors.

## Example 4

The following is a list of constructs which may be used in the methods of the present invention. The vector pBabe.puro, which is used as a starting material to produce many of the below listed constructs, was originally constructed and reported by Morgenstern, J. P. and H. Land, 1990 *Nucl. Acids Res.* 18(12):3587-3596, which is incorporated herein by reference. The pBabe.puro plasmid is particularly useful for expression of exogenous genes in mammalian cells. DNA sequences to be expressed are inserted at cloning sites under the control of the Moloney murine leukemia virus (Mo MuLV) long terminal repeat (LTR) promoter. The plasmid contains the selectable marker for puromycin resistance.

## Example 5

Plasmid pBa.Vo3 is a 7.8 kb plasmid that contains a 2.7 kb EcoRI genomic fragment encoding the T cell receptor Va3 region containing the L, V and J segments cloned into the EcoRI site of pBabe.puro. The T cell receptor-derived target protein is useful in the immunization against and treatment of T cell mediated autoimmune disease and clonotypic T cell lymphoma and leukemia.

## Example 6

Plasmid pBa.gagpol-vpr is a 9.88 kb plasmid that contains the gag/pol and vif genes from HIV/MN cloned into pBabe.puro. The vpr gene is deleted. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. The HIV DNA sequence is published in Reiz, M. S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.: M17449, which is incorporated herein by reference.

## Example 7

Plasmid pM160 is an 11.0 kb plasmid that contains the 2.3 kb PCR fragment encoding the HIV-1/3B envelope protein and rev/tat genes cloned into pMAMneoBlue. The nef region is deleted. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. The DNA sequence of HIV-1/3B is published in Fisher, A., 1985 *Nature* 316:262, which is incorporated herein by reference. The sequence is accessible from Genbank No.: K03455, which is incorporated herein by reference.

## 34

## Example 8

Plasmid pBa.VL is a 5.4 kb plasmid that contains PCR fragment encoding the VL region of an anti-DNA antibody cloned into pBabe.puro at the XbaI and EcoRI sites. The antibody-derived target protein is an example of a target protein useful in the immunization against and treatment of B cell mediated autoimmune disease and clonotypic B cell lymphoma and leukemia. A general method for cloning functional variable regions from antibodies can be found in Chaudhary, V. K., et al., 1990 *Proc. Natl. Acad. Sci. USA* 87:1066, which is incorporated herein by reference.

## Example 9

Plasmid pOspA.B is a 6.84 kb plasmid which contains the coding regions encoding the OspA and OspB antigens of the *Borrelia burgdorferi*, the spirochete responsible for Lyme's disease cloned into pBabe.puro at the BamHI and SalI sites. The PCR primers used to generate the OspA and OspB fragments are 5'-GAAGGATCCATGAAAAAATATTTAT-TGGG-3' (SEQ ID NO:3) and 5'-ACTGTCGACTTATTT-TAAAGCGTTTAAAG-3' (SEQ ID NO: 4). See: Williams, W. V., et al. 1992 *DNA and Cell. Biol.* 11(3):207, which is incorporated herein by reference. The plasmid which contains these pathogen genes, which encode target proteins, is useful in the immunization against Lyme's disease.

## Example 10

Plasmid pBa. Rb-G is a 7.10 kb plasmid which contains a PCR generated fragment encoding the rabies G protein cloned into pBabe.puro at the BamHI site. The plasmid which contains this pathogen gene, which encodes the rabies G protein, is useful in the immunization against Rabies. The DNA sequence is disclosed in Genebank No.:M32751, which is incorporated herein by reference. See also: Anilionis, A., et al., 1981 *Nature* 294:275, which is incorporated herein by reference.

## Example 11

Plasmid pBa. HPV-L1 is a 6.80 kb plasmid which contains a PCR generated fragment encoding the L1 capsid protein of the human papillomavirus (HPV) including HPV strains 16, 18, 31 and 33 cloned into pBabe.puro at the BamHI and EcoRI sites. The plasmid is useful in the immunization against HPV infection and the cancer caused thereby. The DNA sequence is disclosed in Genebank No.:M15781, which is incorporated herein by reference. See also: Howley, P., 1990 *Fields Virology*, Volume 2, Eds.: Channock, R. M. et al. Chapter 58:1625; and Shah, K. and P. Howley, 1990 *Fields Virology*, Volume 2, Eds.: Channock, R. M. et al. Chapter 59; both of which are incorporated herein by reference.

## Example 12

Plasmid pBa.HPV-L2 is a 6.80 kb plasmid which contains a PCR generated fragment encoding the L2 capsid protein of the human papillomavirus (HPV) including HPV strains 16, 18, 31 and 33 cloned into pBabe.puro at the BamHI and EcoRI sites. The plasmid is useful in the immunization against HPV infection and the cancer caused thereby. The DNA sequence is disclosed in Genebank No.:M15781, which is incorporated herein by reference. See also: Howley, P., 1990 *Fields Virology*, Volume 2, Eds.: Channock, R. M. et al. Chapter 58:1625; and Shah, K. and P. Howley, 1990 *Fields Virology*, Volume 2, Eds.: Channock, R. M. et al.

Chapter 59; both of which are incorporated herein by reference.

#### Example 13

Plasmid pBa.MNp7 is a 5.24 kb plasmid which contains a PCR generated fragment encoding the p7 coding region including the HIV MN ag (core protein) sequence cloned into pBabe.puro at the Bam HI site. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M. S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.:M17449, which is incorporated herein by reference.

#### Example 14

Plasmid pGA733-2 is a 6.3 kb plasmid that contains the GA733-2 tumor surface antigen cloned from the colorectal carcinoma cell line SW948 into pCDM8 vector (Seed, B. and A. Aruffo, 1987 *Proc. Natl. Acad. Sci. USA* 84:3365, which is incorporated herein by reference) at BstXI site. The tumor-associated target protein is an example of a target protein useful in the immunization against and treatment of hyperproliferative disease such as cancer. The GA733-2 antigen is a useful target antigen against colon cancer. The GA733 antigen is reported in Szala, S. et al., 1990 *Proc. Natl. Acad. Sci. USA* 87:3542-3546, which is incorporated herein by reference.

#### Example 15

Plasmid pT4-pMV7 is a 11.15 kb plasmid that contains cDNA which encodes human CD4 receptor cloned into pMV7 vector at the EcoRI site. The CD4 target protein is useful in the immunization against and treatment of T cell lymphoma. Plasmid pT4-pMV7 is available from the AIDS Repository, Catalog No. 158.

#### Example 16

Plasmid pDJGA733 is a 5.1 kb plasmid that contains the GA733 tumor surface antigen cloned into pBabe.puro at the BamHI site. The tumor-associated target protein is an example of a target protein useful in the immunization against and treatment of hyperproliferative disease such as cancer. The GA733 antigen is a useful target antigen against colon cancer.

#### Example 17

Plasmid pBa.RAS is a 6.8 kb plasmid that contains the ras coding region that was first subcloned from pZIPneoRAS and cloned into pBabe.puro at the BamHI site. The ras target protein is an example of a cytoplasmic signalling molecule. The method of cloning ras is reported in Weinberg 1984 *Mol. Cell. Biol.* 4:1577, which is incorporated herein by reference. Ras encoding plasmid are useful for the immunization against and treatment of hyperproliferative disease such as cancer; in particular, ras related cancer such as bladder, muscle, lung, brain and bone cancer.

#### Example 18

Plasmid pBa.MNp55 is a 6.38 kb plasmid which contains a PCR generated fragment encoding the p55 coding region including the HIV MN gag precursor (core protein) sequence cloned into pBabe.puro at the BamHI site. The plasmid which contains these HIV viral genes, which encode

HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M. S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.:M17449, which is incorporated herein by reference.

#### Example 19

Plasmid pBa.MNp24 is a 5.78 kb plasmid which contains a PCR generated fragment from the pMN-SF1 template encoding the p24 coding region including the whole HIV MN gag coding region cloned into pBabe.puro at the BamHI and EcoRI sites. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M. S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.: M17449, which is incorporated herein by reference.

#### Example 20

Plasmid pBa.MNp17 is a 5.5 kb plasmid which contains a PCR generated fragment encoding the p17 coding region including the HIV MN gag (core protein) sequence cloned into pBabe.puro at the BamHI and EcoRI sites. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M. S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.: M17449, which is incorporated herein by reference.

#### Example 21

Plasmid pBa.SIVenv is a 7.8 kb plasmid which contains a 2.71 PCR generated fragment amplified from a construct containing SIV 239 in pBR322 cloned into pBabe.puro at the BamHI and EcoRI sites. The primers used are 5'-GC-CAGTTTGGATCCTTAAAAAGGCTTGG-3' (SEQ ID NO:5) and 5'-TTGTGAGGGACAGAATTCCAAT-CAGGG-3' (SEQ ID NO:6). The plasmid is available from the AIDS Research and Reference Reagent Program; Catalog No. 210.

#### Example 22

Plasmid pcTSP/ATK.env is a 8.92 kb plasmid which contains a PCR generated fragment encoding the complete HTLV envelope coding region from HTLV-1/TSP and /ATK isolates subcloned into the pcDNA1/neo vector. The primers used are 5'-CAGTGATATCCCGGAGACTCCTC-3' (SEQ ID NO:7) and 5'-GAATAGAAGAACTCCTCTAGAATTCT-3' (SEQ ID NO:8). Plasmid pcTSP/ATK.env is reported in 1988 *Proc. Natl. Acad. Sci. USA* 85:3599, which is incorporated herein by reference. The HTLV env target protein is useful in the immunization against and treatment of infection by HTLV and T cell lymphoma.

#### Example 23

Plasmid pBa.MNp160 is a 7.9 kb plasmid which contains a 2.8 kb PCR generated fragment amplified from a construct containing MNenv in pSP72 and cloned into pBabe.puro at the BamHI and EcoRI sites. The primers used are 5'-GCCTTAGGCGGATCCTATGGCAGGAAG-3' (SEQ ID NO:9) and 5'-TAAGATGGGTGGCCATGGT-

## 37

GAATT-3' (SEQ ID NO:10). Reiz, M. S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.: M17449, which is incorporated herein by reference. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS.

## Example 24

Plasmid pC.MNP55 is a 11.8 kb plasmid which contains a 1.4 kb PCR generated fragment amplified from the gag region of MN isolate and cloned into the pCEP4 vector. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS. Reiz, M. S., 1992 *AIDS Res. Human Retro.* 8:1549, which is incorporated herein by reference. The sequence is accessible from Genbank No.: M17449, which is incorporated herein by reference.

## Example 25

Plasmid pC.Neu is a 14.2 kb plasmid that contains a 3.8 kb DNA fragment containing the human neu oncogene coding region that was cut out from the LTR-2/erbB-2 construct and subcloned into the pCEP4 vector. The pC.Neu plasmid is reported in DiFiore 1987 *Science* 237:178, which is incorporated herein by reference. The neu oncogene target protein is an example of a growth factor receptor useful as a target protein for the immunization against and treatment of hyperproliferative disease such as cancer; in particular, colon, breast, lung and brain cancer.

## Example 26

Plasmid pC.RAS is a 11.7 kb plasmid that contains a 1.4 kb DNA fragment containing the ras oncogene coding region that was first subcloned from pZIPneoRAS and subcloned into pCEP4 at the BamHI site. The pC.RAS plasmid is reported in Weinberg 1984 *Mol. Cell. Biol.* 4:1577, which is incorporated herein by reference. The ras target protein is an example of a cytoplasmic signalling molecule. Ras encoding plasmid are useful for the immunization against and treatment of hyperproliferative disease such as cancer; in particular, ras related cancer such as bladder, muscle, lung, brain and bone cancer.

## Example 27

Plasmid pNLpuro is a 15 kb plasmid which contains HIV gag/pol and SV40-puro insertion. The plasmid which contains these HIV viral genes, which encode HIV target proteins, is useful in the immunization against and treatment of HIV infection and AIDS.

## Example 28

A DNA construct was designed to test the effectiveness of a genetic vaccine against human CD4 in mice. These experiments were designed to test the ability of a vaccine to protect against a T lymphoma antigen. In T cell lymphoma, CD4 is a tumor specific antigen. Accordingly, this model demonstrates the ability of the genetic vaccine to protect against T lymphoma. Further, these experiments tested the effectiveness against a member of the immunoglobulin superfamily of molecules. CD4 is highly conserved between human and murine species.

## 38

The animal model used was described above. Tumor cells were transfected with DNA encoding CD4, confirmed to express the protein and implanted into the animal. Controls included untransfected tumor lines. Although the animals were immunocompetent, an immune response was not directed against the implanted, CD4-labelled tumors in unvaccinated animals.

Genetically immunized animals were vaccinated with plasmid pT4-pMVT, a 11.15 kb plasmid that contains cDNA which encodes human CD4 receptor cloned into pMV7 vector at the EcoRI site. Plasmid pT4-pMV7 is available from the AIDS Repository, Catalog No. 158. Controls included unvaccinated animals and animals administered the CD4 protein.

In the genetic immunization procedure described herein, the quadriceps muscles of BALB/c mice were injected with 100  $\mu$ l of 0.5% bupivacaine-HCl and 0.1% methylparaben in isotonic NaCl using a 27-gauge needle to stimulate muscle cell regeneration to facilitate uptake of the genetic construct. Twenty-four hours later, the same injection sites were then injected with either 100  $\mu$ g of pT4-pMV7 or with 100  $\mu$ g of pMV7 as a control plasmid. The mice were boosted by injecting the same amount of DNA construct three times at two week intervals in the same manner but without pre-treatment with bupivacaine-HCl.

Animals received 1,000,000 CD4-labelled tumor cells. In non-vaccinated animals, large tumors formed and death resulted after about 7-10 weeks. Vaccinated animals did not develop similar deadly tumors.

Results demonstrate that the immune response of genetically vaccinated mice was sufficient to completely eliminate the transfected tumors while having no effect on untransfected tumors. CD4 protein vaccination led to some reduction in tumor size in transfected tumors as compared to untransfected tumors but had no effect on mortality. Unvaccinated animals showed similar mortality for both transfected and untransfected tumors.

## Example 29

A DNA construct was designed to test the effectiveness of a genetic vaccine against human GA733 in mice. These experiments were designed to test the ability of a vaccine to protect against GA733 associated cancer such as colon cancer. The animal model used was described above. Tumor cells were transfected with DNA encoding GA733, confirmed to express the protein and implanted into the animal. Controls included untransfected tumor lines.

Genetically immunized animals were vaccinated with plasmid pGA733-2, a 6.3 kb plasmid that contains the GA733-2 tumor surface antigen cloned from the colorectal carcinoma cell line SW948 into pCDM8 vector at BstXI site following the method described above. Controls included unvaccinated animals and animals administered the GA733 protein.

Results demonstrate that the immune response of genetically vaccinated mice was sufficient to completely eliminate the transfected tumors while having no effect on untransfected tumors. GA733 protein vaccination led to some reduction in tumor size in transfected tumors as compared to untransfected tumors but had no effect on mortality. Unvaccinated animals showed similar mortality for both transfected and untransfected tumors.

## Example 30

A DNA construct was designed to test the effectiveness of a genetic vaccine against human p185neu in mice. These

39

experiments were designed to test the ability of a vaccine to protect against p185neu associated cancer such as breast, lung and brain cancer. The animal model used was described above. Tumor cells were transfected with DNA encoding neu, confirmed to express the protein and implanted into the animal. Controls included untransfected tumor lines.

Genetically immunized animals were vaccinated with plasmid pLTR-2/erbB-2, a 14.3 kb plasmid that contains the human neu oncogene coding region cloned into the LTR-2 vector at the XhoI site following the method described above. The 5'LTR and 3'LTR are from Moloney-MuLV LTR. Controls included unvaccinated animals and animals administered the p185neu protein.

Results demonstrate that the immune response of genetically vaccinated mice was sufficient to completely eliminate the transfected tumors while having no effect on untransfected tumors. p185 protein vaccination led to some reduction in tumor size in transfected tumors as compared to untransfected tumors but had no effect on mortality. Unvaccinated animals showed similar mortality for both transfected and untransfected tumors.

#### Example 31

A DNA construct was designed to test the effectiveness of a genetic vaccine against human Ras in mice. These experiments were designed to test the ability of a vaccine to protect against Ras associated cancer such as bladder, muscle, lung, brain and bone cancer. The animal model used was described above. Tumor cells were transfected with DNA encoding Ras, confirmed to express the protein and implanted into the animal. Controls included untransfected tumor lines.

Genetically immunized animals were vaccinated with plasmid pBa.RAS is a 6.8 kb plasmid that contains the ras coding region that was first subcloned from pZIPneoRAS and cloned into pBabe.puro at the BamHI site following the vaccination method described above. The ras target protein is an example of a cytoplasmic signalling molecule. The method of cloning ras is reported in Weinberg 1984 *Mol. Cell. Biol.* 4:1577, which is incorporated herein by reference. Controls included unvaccinated animals and animals administered the Ras protein.

#### Example 32

A DNA construct was designed to test the effectiveness of a genetic vaccine against human rabies G protein antigen in mice. The animal model used was described above. Tumor cells were transfected with DNA encoding rabies G protein, confirmed to express the protein and implanted into the animal. Controls included untransfected tumor lines.

Genetically immunized animals were vaccinated with plasmid pBa. Rb-G is a 7.10 kb plasmid which contains a PCR generated fragment encoding the rabies G protein cloned into pBabe.puro at the BamHI site, following the vaccination method described above. The rabies G target protein is an example of a pathogen antigen. The DNA sequence is disclosed in Genebank No. M32751. Controls included unvaccinated animals and animals administered the G protein.

#### Example 33

A DNA construct was designed to test the effectiveness of a genetic vaccine against Lyme's disease antigen in mice. The animal model used was described above. Tumor cells

40

were transfected with DNA encoding OspA and Osp B, confirmed to express the protein and implanted into the animal. Controls included untransfected tumor lines.

Genetically immunized animals were vaccinated with plasmid pOspA.B is a 6.84 kb plasmid which contains the coding regions encoding the OspA and Osp. B antigens of the *Borrelia burgdorferi*, the spirochete responsible for Lyme's disease cloned into pBabe.puro at the BamHI and SalI sites, following the vaccination method described above. The OspA and OspB target proteins are examples of pathogen antigens. The PCR primers used to generate the OspA and OspB fragments are 5'-GAAGGATCCAT-GAAAAAATATTATTGGG-3' (SEQ ID NO:3) and 5'-ACTGTGCGACTTATTTTAAAGCGTTTTTAAAG-3' (SEQ ID NO: 4). See: Williams, W. V., et al. 1992 *DNA and Cell. Biol.* 11(3):207, which is incorporated herein by reference. Controls included unvaccinated animals and animals administered OspA and OspB proteins.

#### Example 34

A DNA construct was designed to test the effectiveness of a genetic vaccine against a human T cell receptor variable region in mice. These experiments were designed to test the ability of a vaccine to protect against a T cell receptor derived protein associated cancer such as T cell lymphoma and T cell mediated autoimmune disease. The animal model used was described above. Tumor cells were transfected with DNA encoding Ras, confirmed to express the protein and implanted into the animal. Controls included untransfected tumor lines.

Genetically immunized animals were vaccinated with plasmid pBa.Vα3 is a 7.8 kb plasmid that contains a 2.7 kb EcoRI genomic fragment encoding the T cell receptor Vα3 region containing the L, V and J segments cloned into the EcoRI site of pBabe.puro following the vaccination method described above.

#### Example 35

The plasmid pM160 can be used as a starting material for several plasmids useful to express one or more genes from the env portion of HIV. As described above, the DNA segment encoding the envelope gene of HIV-1 HXB2 was cloned by the polymerase chain reaction (PCR) amplification technique utilizing the lambda cloned DNA obtained from the AIDS Research and Reference Reagent Program. The sequences of the 5' and 3' primers are 5'-AGGCGTCTC-GAGACAGAGGAGAGCAAGAAATG-3' (SEQ ID NO:1) with incorporation of XhoI site and 5'-TTTCCCTCTA-GATAAGCCATCCAATCACAC-3' (SEQ ID NO: 2) with incorporation of XbaI site, respectively, which encompass gp160, tat and rev coding region. The nef gene is absent. Gene specific amplification was performed using Taq DNA polymerase according to the manufacturer's instructions (Perkin-Elmer Cetus Corp.). The PCR reaction products were treated with 0.5 ug/ml proteinase K at 37° C. for thirty minutes followed by a phenol/chloroform extraction and ethanol precipitation. Recovered DNA was then digested with XhoI and XbaI for two hours at 37° C. and subjected to agarose gel electrophoresis. The isolated and purified XhoI-XbaI PCR fragment was cloned into Bluescript plasmid (Stratagene Inc., La Jolla, Calif.) and then subcloned into the eukaryotic expression vector pMAMneoBlue (Clontech Laboratories, Inc., Palo Alto, Calif.). The resulting construct was designated as pM160. The plasmid DNA was purified with CsCl gradient ultracentrifugation. The restriction

enzyme map for pMAMneoBlue plasmid is available from the manufacturer and may be used by those having ordinary skill in the art to engineer, that is to change, delete and add various elements using standard molecular biology techniques and widely available starting material.

The promoter controlling gp160/rev/tat gene expression is MMTV LTR. The promoter may be deleted and replaced with Actin promoter, myosin promoter, HIV LTR promoter and CMV promoter.

The gene conferring ampicillin resistance may be deleted or otherwise inactivated. The gene conferring neomycin resistance may be placed under the control of a bacterial promoter.

The Rous sarcoma virus enhancer may be deleted from the plasmid. The RSV enhancer may be replaced with the muscle creatine enhancer.

The gp160/rev/tat genes overlap and share the same nucleotide sequences in different reading frames. The rev gene may be deleted by changing its initiation codon to a different codon. Similarly, the tat gene may be eliminated by the same means. In each plasmid except those using the HIV LTR promoter to control gp160/rev/tat, either rev, tat, or both rev and tat may be eliminated. In plasmids using the HIV LTR promoter, tat must be present.

The following Table lists pM160-modified plasmids. Each plasmid has an inactivated ampicillin gene. Each has deleted the RSV enhancer. Some have no enhancer (no); some have creatine muscle enhancer (CME). Some have the HIV rev gene (yes) while it is deleted in others (no). Some have the HIV tat gene (yes) while it is deleted in others (no).

Construct	Promoter	enhancer	rev	tat
RA-1	Actin	no	yes	yes
RA-2	Actin	no	yes	no
RA-3	Actin	no	no	yes
RA-4	Actin	CME	yes	yes
RA-5	Actin	CME	yes	no
RA-6	Actin	CME	no	yes
RA-7	CMV	no	yes	yes
RA-8	CMV	no	yes	no
RA-9	CMV	no	no	yes
RA-10	CMV	CME	yes	yes
RA-11	CMV	CME	yes	no
RA-12	CMV	CME	no	yes
RA-13	MMTV	no	yes	yes
RA-14	MMTV	no	yes	no
RA-15	MMTV	no	no	yes
RA-16	MMTV	CME	yes	yes
RA-17	MMTV	CME	yes	no
RA-18	MMTV	CME	no	yes
RA-19	Myosin	no	yes	yes
RA-20	Myosin	no	yes	no
RA-21	Myosin	no	no	yes
RA-22	Myosin	CME	yes	yes
RA-23	Myosin	CME	yes	no
RA-24	Myosin	CME	no	yes
RA-25	HIV-1 LTR	no	yes	yes
RA-26	HIV-1 LTR	no	no	yes
RA-27	HIV-1 LTR	CME	yes	yes
RA-28	HIV-1 LTR	CME	no	yes

Constructions RA-29 to RA-56 are identical to RA-1 to RA-32 respectively except in each case the promoter controlling the neomycin gene is a bacterial promoter.

### Example 36

The plasmid pNLpuro may be used as a starting material to produce several different plasmids which express the HIV gag/pol genes. As described above, pNLpuro was con-

structed for expression of gag pol. The HIV-1 genomic clone pNL43 was obtained through the NIH AIDS Research and Reference Reagent Program (ARRRP), Division of AIDS, NIAID, NIH, from Dr. Malcom Martin. The pNL43 clone is a construct that consists of HIV-1 proviral DNA plus 3 kb of host (i.e. human) sequence from the site of integration (5' and 3' of the HIV sequence) cloned into pUC18. The StuI site within the non-HIV 5' flanking human DNA of pNL43 was destroyed by partial digestion with StuI followed by digestion of the free ends with *E. coli* polymerase I. The linear plasmid was filled and then self ligated, leaving a unique StuI site within the HIV genome. This plasmid, pNLDstu, was then digested with the blunting enzymes StuI and BsaBI which eliminated a large section of the coding sequence for gp120. The SV40 promoter and puromycin resistance coding region (puromycin acetyl transferase (PAC)) were isolated from pBABE-puro (Morgenstern and Land, 1990 *Nucl. Acids Res.* 18(12):3587-3596, which is incorporated herein by reference, kindly provided by Dr. Hartmut Land of the Imperial Cancer Research Fund) using EcoRI and ClaI. This fragment was blunted, then cloned into the StuI/BsaBI-digested pNLDstu. A clone was selected with the SV40-puro fragment in the correct orientation so that the 3' LTR of HIV could provide poly A functions for the PAC message. This plasmid was designated pNLpuro.

The vpr regulatory gene is deleted from the HIV gag pol vector in order to eliminate a necessary regulatory protein from the set of genes to be introduced by vaccination. A region from just upstream of the unique PstMI site to just after the vif termination codon was amplified via PCR using primers that introduced a non-conservative amino acid change (glu→val) at amino acid 22 of vpr, a stop codon in the vpr reading frame immediately after amino acid 22, and an EcoRI site immediately following the new stop codon. This PCR fragment was substituted for the PstMI-EcoRI fragment of pNLpuro or pNL43. This substitution resulted in the deletion of 122 nucleotides of the open reading frame of vpr, thus eliminating the possibility of reversion that a point mutation strategy entails. The resulting plasmids, pNLpuroΔvpr, encode the first 21 natural amino acids of vpr plus a valine plus all other remaining HIV-1 genes and splice junctions in their native form. Such deletion strategy would also be applicable to nef, vif, and vpu and allow for structural gene expression but protect from the generation of a live recombinant virus.

In addition to vpr, other changes may be made by those having ordinary skill in the art to plasmid pNL43puro using standard molecular biology techniques and widely available starting material.

The human flanking sequences 5' and 3' of the HIV sequences can be removed by several methods. For example, using PCR, only HIV, SV40-puro, and pUC18 sequences can be amplified and reconstructed.

The psi region of HIV, which is important in the packaging of the virus, can be deleted from pNL43puro-based plasmids. In order to delete the psi region, the pNLpuro plasmid is cut with SacI and SpeI. This digestion removes the psi region as well as the 5' LTR which is upstream and portion of the gag/pol region which is downstream of psi. In order to reinsert the deleted non-psi sequences, PCR amplification is performed to regenerate those sequences. Primers are designed which regenerate the portions of the HIV sequence 5' and 3' to psi without regenerating psi. The primers reform the SacI site at the portion of the plasmid 5' of the 5' LTR. Primers go downstream from a site upstream of the SacI site to a site just 3' of the 5' end of the psi region, generating an AatI site at the 3' end. Primers starting just 5'

43

of the psi region also generate an AaII site and, starting 3' of the SpeI site, regenerate that site. The PCR generated fragments are digested with SacI, AaII and SpeI and ligated together with the SacI/SpeI digested pHLpuro-psi-fragment. The HIV 5'LTR promoter can be deleted and replaced with Moloney virus promoter, MMTV LTR, Actin promoter, myosin promoter and CMV promoter.

The HIV 3'LTR polyadenylation site can be deleted and replaced with SV40 polyadenylation site.

The gene conferring ampicillin resistance may be deleted or otherwise inactivated.

The following is a list of pNLpuro-based constructions in which HIV psi and vpr regions are deleted and human flanking regions 5' and 3' of the HIV sequences are deleted.

Construct	Promoter	poly(A)	Amp <sup>r</sup>
LA-1	Moloney	HIV 3' LTR	yes
LA-2	Moloney	SV40	yes
LA-3	Moloney	HIV 3' LTR	no
LA-4	Moloney	SV40	no
LA-5	CMV	HIV 3' LTR	yes
LA-6	CMV	SV40	yes
LA-7	CMV	HIV 3' LTR	no
LA-8	CMV	SV40	no
LA-9	MMTV	HIV 3' LTR	yes
LA-10	MMTV	SV40	yes
LA-11	MMTV	HIV 3' LTR	no
LA-12	MMTV	SV40	no
LA-13	HIV 5' LTR	HIV 3' LTR	yes
LA-14	HIV 5' LTR	SV40	yes
LA-15	HIV 5' LTR	HIV 3' LTR	no
LA-16	HIV 5' LTR	SV40	no

Constructions LA-17 to LA-32 are identical to LA-1 to LA-16 respectively except in each case at least one of the human flanking sequence remains.

#### Example 37

In another construction for expressing the env gene, that region of HIV may be inserted into the commercially available plasmid pCEP4 (Invitrogen). The pCEP4 plasmid is particularly useful since it contains the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration. pCEP4 also contains the hygromycin marker under the regulatory control of the thymidine kinase promoter and polyadenylation site. The HIV env coding region is placed under the regulatory control of the CMV promoter and SV40 polyadenylation site. The HIV env coding region was obtained as a 2.3 kb PCR fragment from HIV/3B, Genebank sequence K03455. The resulting pCEP4-based plasmid, pRA-100, is maintained extrachromosomally and produces gp160 protein.

#### Example 38

In another construction for expressing the env gene, that region of HIV may be inserted into the commercially available plasmid pREP4 (Invitrogen). The pREP4 plasmid is particularly useful since it contains the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration. pREP4 also contains the hygromycin marker under the regulatory control of the thymidine kinase promoter and polyadenylation site. The HIV env coding region is placed under the regulatory control of the RSV promoter and SV40 polyadenylation site. The HIV env coding region

44

was obtained as a 2.3 kb PeR fragment from HIV/3B, Genebank sequence K03455. The resulting pCEP4-based plasmid, pRA-101, is maintained extrachromosomally and produces gp160 protein.

#### Example 39

In another construction for expressing the gag/pol genes, that region of HIV may be inserted into the commercially available plasmid pCEP4 (Invitrogen). The pCEP4 plasmid is particularly useful since it contains the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration. pCEP4 also contains the hygromycin marker under the regulatory control of the thymidine kinase promoter and polyadenylation site. The HIV gag/pol coding region is placed under the regulatory control of the CMV promoter and SV40 polyadenylation site. The HIV gag/pol coding region was obtained from HIV MN, Genebank sequence MI7449, and includes the vif gene. The vpr gene is not included. The resulting pCEP4-based plasmid, pLA-100, is maintained extrachromosomally and produces GAG55, reverse transcriptase, protease and integrase proteins.

#### Example 40

In another construction for expressing the gag/pol genes, that region of HIV may be inserted into the commercially available plasmid pREP4 (Invitrogen). The pREP4 plasmid is particularly useful since it contains the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region which produces high copy episomal replication without integration. pREP4 also contains the hygromycin marker under the regulatory control of the thymidine kinase promoter and polyadenylation site. The HIV gag/pol coding region is placed under the regulatory control of the CMV promoter and SV40 polyadenylation site. The HIV gag/pol coding region was obtained from HIV MN, Genebank sequence MI7449, and includes the vif gene. The vpr gene is not included. The resulting pREP4-based plasmid, pLA-101, is maintained extrachromosomally and produces GAG55, reverse transcriptase, protease and integrase proteins.

#### Example 41

The following construction, referred to herein as pGAGPOL.rev, is useful to express HIV gag/pol genes.

The plasmid includes a Kanamycin resistance gene and a pBR322 origin of DNA replication. The sequences provided for transcription regulation include: a cytomegalovirus promoter; a Rous sarcoma virus enhancer; and an SV40 polyadenylation signal. The HIV-1 sequences included in pGAGPOL.rev include a sequence that encodes gag; a sequence that encodes pol; a sequence that encodes reverse transcriptase which contains a small deletion; a sequence that encodes the inactive amino terminus of Int; and a sequence that encodes rev. Each of the HIV sequences are derived from HIV-1 strain HXB2.

Several safety features are included in pGAGPOL.rev. These include use of the CMV promoter and a non-retroviral poly(A) site. Furthermore, deletion of the  $\psi$  sequence limits the ability to package viral RNA. In addition, multiple mutations of the reverse transcriptase yield an enzymatically inactive product. Moreover, a large deletion of integrase yields an inactive product and a Kanamycin resistance marker is used for stabilizing bacterial transformants.

Plasmid pGAGPOL.rev is constructed as follows.

Step 1. A subclone of part of the HIV-1 (HXB2) genome that is cloned into Bluescript (Stratagene) is used. The subclone of HIV-1 contains the complete 5'LTR and the rest of the HIV-1 genome to nucleotide 5795 (Genebank numbering). The HIV-1 sequences are obtained from the HXB2D plasmid (AIDS Repository).

Step 2. PCR part of gag from the open reading frame HXB2D plasmid (AIDS Repository). Cut PCR fragment with NotI and SpeI and ligate with HIV-1 subclone described above restricted with NotI and SpeI.

Step 3. PCR gag/pol junction and part of pol-encoding sequences from the HXB2D plasmid (AIDS Repository) with primers SEQ ID NO.:17 and SEQ ID NO.:18. Cut PCR product with ClaI and ligate together. Cut ligated fragments with BclI and SalI and ligate with plasmid from Step 2 digested with BclI and SalI.

Step 4. Cut plasmid from Step 3 with BspMI and EcoRI and religate with adapters formed by annealing linkers SEQ ID NO.:19 and SEQ ID NO.:20.

Step 5. Cut plasmid from Step 4 with NotI and SalI and ligate with plasmid from either 4a or 4b in description written for pENV (below). Cut also with NotI and SalI.

Step 6. Restrict plasmid from Step 5 with SalI and MluI and ligate with PCR product obtained by PCR of rev with primers SEQ ID NO.:21 and SEQ ID NO.:22.

Step 7. Cut plasmid from Step 6 with NotI and ligate with product obtained by PCR of the rev responsive element in the HXB2D plasmid (AIDS Repository) with primers SEQ ID NO.:23 and SEQ ID NO.:24.

Steps 6 and 7 are optional.

#### Example 42

The following construction, referred to herein as pENV, is useful to express HIV env genes.

The plasmid includes a Kanamycin resistance gene and a pBR322 origin of DNA replication. The sequences provided for transcription regulation include: a cytomegalovirus promoter; a Rous sarcoma virus enhancer; and an SV40 polyadenylation signal. The HIV-1 sequences included in pENV include a sequence that encodes vpu; a sequence that encodes rev; a sequence that encodes gp160; a sequence that encodes 50% of nef; a sequence that encodes vif; and, a sequence that encodes vpr with a 13 amino acid carboxy-end deletion. The vpu, rev, gp160 and nef sequences are derived from HIV-1 strain MN. The vif and vpr sequences are derived from HIV-1 strain HXB2.

Several safety features are included in pGAGPOL.rev. These include use of the CMV promoter and a non-retroviral poly(A) site. Furthermore, tat has been deleted and a 50% deletion of nef yields an "inactive" nef product. In addition, vif and vpr are placed out of normal sequence and a partial deletion of vpr further ensures an inactive vpr product.

Plasmid pENV is constructed as follows.

Step 1. Start with pUC18 digested with HindIII and EcoRI. The resulting fragment that contains the ColE1 origin of replication and the lacI gene should be ligated with the EcoRI/HindIII fragment from pMAMneoBlue that contains the Rous sarcoma virus enhancer. The resulting plasmid or pMAMneo-Blue from Clontech (Palo Alto, Calif.) can then be digested with HindIII and BglII. Using standard techniques, ligate with fragment containing kn gene obtained by PCR of geneblock plasmid (Pharmacia).

Step 2. If pMAMneo-Blue used as starting plasmid, digest with MluI and EcoRI, fill in the ends with Klenow fragment of Polymrase I and religate.

Step 3. Then, with either pMAMneo-Blue or pUC18-derived plasmid, digest with HindIII and ligate with the SV40 polyA site and early splicing region obtained by PCR of pCEP4 (Invitrogen, San Diego Calif.) with primers SEQ ID NO.:25 and SEQ ID NO.:26.

Step 4a. Digest with BamHI and ligate with the CMV promoter obtained by PCR of pCEP4 (Invitrogen, San Diego Calif.) with primers SEQ ID NO.:27 and SEQ ID NO.:28.

Step 4b. Digest with BamHI and ligate with the MoMLV LTR obtained by PCR with primers SEQ ID NO.:29 and SEQ ID NO.:30.

Step 5. Digest with NotI and MluI and ligate with GP160 coding region obtained by PCR of pMN-ST1 with primers SEQ ID NO.:31 and SEQ ID NO.:32.

Step 6. Digest with MluI and ligate with sequences that encode vif in its entirety and vpr with a 13aa carboxy-end deletion by CPR of HXB2D plasmid (AIDS Repository) with primers SEQ ID NO.:33 and SEQ ID NO.:34.

#### Example 43

An immunization system is provided which comprises:

a pharmaceutical composition comprising about 100 µg of pGAGPOL. rev in an isotonic, pharmaceutically acceptable solution; and,

a pharmaceutical preparation comprising 100 µg of pENV in an isotonic, pharmaceutically acceptable solution. In addition, the immunization system preferably comprises a pharmaceutical composition comprising about 1 ml of 0.5% bupivacaine-HCl and 0.1% methylparaben in an isotonic pharmaceutical carrier.

In such a preferred immunization system, a first set of administrations is performed in which bupivacaine and one of the two pharmaceutical compositions are administered intramuscularly to an individual, preferably into a muscle of an arm or buttock. Bupivacaine and the other of the two pharmaceutical compositions are administered intramuscularly to the individual at a different site, preferably remote from the site of the administration of the one pharmaceutical composition, preferably into a muscle of the other arm or buttock. Subsequence sets of administrations may be performed later in time, preferably 48 hours to two weeks or more later.

The immunization system may be used to vaccinate an individual in order to protect that individual from HIV infection or to treat an HIV infected individual with an immunotherapeutic.

#### Example 44

In some embodiments, the present invention relates to a method of immunizing an individual against HIV by administering a single inoculant. This inoculant includes a genetic construct that comprises at least one, preferably two, more preferably more than two or a plurality of the genes of the HIV virus or all of the structural genes. However, the inoculant does not contain a complete complement of all HIV genes. If a single cell is provided with a complete complement of viral genes, it is possible that a complete infectious virus can be assembled within the cell. Accordingly, a genetic construct according to the present invention is not provided with such a full complement of genes. As a safety precaution, one or more essential genes can be deleted or intentionally altered to further ensure that an infectious viral particle cannot be formed.

In some embodiments of the present invention, at least portions of one, two or all HIV structural genes are provided. The structural genes of HIV consist of gag, pol and env. Portions of at least one of these three genes are provided on a genetic construct. Accordingly, in some embodiments, at least a portion of each of gag and pol are provided on a genetic construct; in some embodiments, at least a portion of env is provided on a genetic construct; in some embodiments, at least a portion of gag is provided on a genetic construct; in some embodiments at least a portion of each of pol and env are provided on a genetic construct; in some embodiments, at least a portion of each of gag and env are provided on a genetic construct; in some embodiments at least a portion of pol is provided on a genetic construct. Optionally, the entire gene is provided. Optionally, in any of these constructs, HIV regulatory genes may also be present. The HIV regulatory genes are: vpr, vif, vpu, nef, tat and rev.

#### Example 45

As used herein, the term "expression unit" is meant to refer to a nucleic acid sequence which comprises a promoter operably linked to a coding sequence operably linked to a polyadenylation signal. The coding sequence may encode one or more proteins or fragments thereof. In preferred embodiments, a expression unit is within a plasmid.

As used herein, the term "HIV expression unit" is meant to refer to a nucleic acid sequence which comprises a promoter operably linked to a coding sequence operably linked to a polyadenylation signal in which the coding sequence encodes a peptide that comprises an epitope that is identical or substantially similar to an epitope found on an HIV protein. "Substantially similar epitope" is meant to refer to an epitope that has a structure which is not identical to an epitope of an HIV protein but nonetheless invokes an cellular or humoral immune response which cross reacts to an HIV protein. In preferred embodiments, the HIV expression unit comprises a coding sequence which encodes one or more HIV proteins or fragments thereof. In preferred embodiments, an HIV expression unit is within a plasmid.

In some embodiments of the present invention, a single genetic construct is provided that has a single HIV expression unit which contains DNA sequences that encode one or more HIV proteins or fragments thereof. As used herein, the term "single HIV expression unit construct" is meant to refer to a single genetic construct that contains a single HIV expression unit. In preferred embodiments, a single HIV expression unit construct is in the form of a plasmid.

In some embodiments of the present invention, a single genetic construct is provided that has more than one HIV expression units in which each contain DNA sequences that encode one or more HIV proteins or fragments thereof. As used herein, the term "multiple HIV expression unit genetic construct" is meant to refer to a single plasmid that contains more than one HIV expression units. In preferred embodiments, a multiple HIV expression unit construct is in the form of a plasmid.

In some embodiments of the present invention, a single genetic construct is provided that has two HIV expression units in which each contain DNA sequences that encode one or more HIV proteins or fragments thereof. As used herein, the term "two HIV expression unit genetic construct" is meant to refer to a single plasmid that contains two HIV expression units, i.e. a multiple HIV expression unit genetic construct that contains two HIV expression unit genetic expression units. In a two HIV expression unit genetic

construct, it is preferred that one HIV expression unit operates in the opposite direction of the other HIV expression unit. In preferred embodiments, a two HIV expression unit construct is in the form of a plasmid.

In some embodiments of the present invention, an HIV genetic vaccine is provided which contains a single genetic construct. The single genetic construct may be a single HIV expression unit genetic construct, a two HIV expression unit genetic construct or a multiple HIV expression unit genetic construct which contains more than two HIV expression units.

In some embodiments of the present invention, an HIV genetic vaccine is provided which contains more than one genetic construct in a single inoculant.

In some embodiments of the present invention, an HIV genetic vaccine is provided which contains more than one genetic construct in more than one inoculant. As used herein, the term "multiple inoculant" is meant to refer to a genetic vaccine which comprises more than one genetic construct, each of which is administered separately. In some embodiments of the present invention, an HIV genetic vaccine is provided which contains two genetic constructs. Each genetic construct may be, independently, a single HIV expression unit genetic construct, a two HIV expression unit genetic construct or a multiple HIV expression unit genetic construct which contains more than two HIV expression units. In some embodiments, both genetic constructs are single HIV expression unit genetic constructs. In some embodiments, both genetic constructs are two HIV expression unit genetic constructs. In some embodiments, both genetic constructs are multiple HIV expression unit genetic constructs. In some embodiments, one genetic construct is a single HIV expression unit genetic construct and the other is a two HIV expression unit genetic construct. One having ordinary skill in the art can readily recognize and appreciate the many variations depending upon the number of genetic constructs used in a genetic vaccine and the number of HIV expression units that may be present on each genetic construct.

It is preferred that the genetic constructs of the present invention do not contain certain HIV sequences, particularly, those which play a role in the HIV genome integrating into the chromosomal material of the cell into which it is introduced. It is preferred that the genetic constructs of the present invention do not contain LTRs from HIV. Similarly, it is preferred that the genetic constructs of the present invention do not contain a psi site from HIV. Further, it is preferred that the reverse transcriptase gene is deleted and the integrase gene is deleted. Deletions include deletion of only some of the codons or replacing some of the codons in order to essentially delete the gene. For example, the initiation codon may be deleted or changed or shifted out of frame to result in a nucleotide sequence that encodes an incomplete and non-functioning.

It is also preferred that the genetic constructs of the present invention do not contain a transcribable tat gene from HIV. The tat gene, which overlaps the rev gene may be completely deleted by substituting the codons that encode rev with other codons that encode the same amino acid for rev but which does not encode the required tat amino acid in the reading frame in which tat is encoded. Alternatively, only some of the codons are switched to either change, i.e. essentially delete, the initiation codon for tat and/or change, i.e. essentially delete, sufficient codons to result in a nucleotide sequence that encodes an incomplete and non-functioning tat.



It is preferred that a genetic construct comprises coding sequences that encode peptides which have at least an epitope identical to or substantially similar to an epitope from HIV gag, pol, env or rev proteins. It is more preferred that a genetic construct comprises coding sequences that encode at least one of HIV gag, pol, env or rev proteins or fragments thereof. It is preferred that a genetic construct comprises coding sequences that encode peptides which have more than one epitopes identical to or substantially similar to an epitope from HIV gag, pol, env or rev proteins. It is more preferred that a genetic construct comprises coding sequences that encode more than one of HIV gag, pol, env or rev proteins or fragments thereof.

In some embodiments, a genetic construct comprises coding sequences that encode peptides which have at least an epitope identical to or substantially similar to an epitope from HIV vif, vpr, vpu or nef proteins. In some embodiments, a genetic construct comprises coding sequences that encode at least one of HIV vif, vpr, vpu or nef proteins or fragments thereof.

A single HIV expression unit genetic construct may comprise coding regions for one or more peptides which share at least one epitope with an HIV protein or fragment thereof in a single expression unit under the regulatory control of single promoter and polyadenylation signal. It is preferred that genetic constructs encode more than one HIV protein or fragment thereof. The promoter may be any promoter functional in a human cell. It is preferred that the promoter is an SV40 promoter or a CMV promoter, preferably a CMV immediate early promoter. The polyadenylation signal may be any polyadenylation signal functional in a human cell. It is preferred that the polyadenylation signal is an SV40 polyadenylation signal, preferably the SV40 minor polyadenylation signal. If more than one coding region is provided in a single expression unit, they may be immediately adjacent to each other or separated by non-coding regions. In order to be properly expressed, a coding region must have an initiation codon and a termination codon.

A two HIV expression unit genetic construct may comprise coding regions for one or more peptides which share at least one epitope with an HIV protein or fragment thereof on each of the two expression units. Each expression unit is under the regulatory control of single promoter and polyadenylation signal. In some embodiments, it is preferred that genetic constructs encode more than one HIV protein or fragment thereof. In some embodiments, it is preferred that nucleotide sequences encoding gag and pol are present on one expression unit and nucleotide sequences encoding env and rev are present on the other. The promoter may be any promoter functional in a human cell. It is preferred that the promoter is an SV40 promoter or a CMV promoter, preferably a immediate early CMV promoter. The polyadenylation signal may be any polyadenylation signal functional in a human cell. It is preferred that the polyadenylation signal is an SV40 polyadenylation signal, preferably the SV40 minor polyadenylation signal. If more than one coding region is provided in a expression unit, they may be immediately adjacent to each other or separated by non-coding regions. In order to be properly expressed, a coding region must have an initiation codon and a termination codon.

According to some embodiments of the present invention, the MHC Class II crossreactive epitope in env is deleted and replaced with the analogous region from HIV II.

When a genetic construct contains gag and/or pol, it is generally important that rev is also present. In addition to rev, a rev response element may be provided with gag and pol for increased expression of those genes.

When genetic constructs are produced that it is preferred that the env gene used in plasmid 1 is derived from MN or MN-like isolates including clinical isolates resembling MN, preferably non-syncytial inducing clinical isolates, preferably those that are macrophage tropic from early stage clinical isolates.

Multiple proteins may be produced from a single expression unit by alternative splicing. Splicing signals are provided to allow alternative splicing which produces different messages encoding different proteins.

#### Example 46

FIG. 8 shows four backbones, A, B, C and D. FIG. 9 shows 4 inserts, 1, 2, 3 and 4. Insert 1 supports expression of gag and pol; the rev response element was cloned in a manner to conserve the HIV splice acceptor. Insert 2 is similar to insert 1 as it too supports expression of gag and pol except the rev response element was cloned without conserving the HIV splice acceptor. Insert 3 supports expression of gag and pol, includes a deletion of the integrase gene and does not include the presence of the cis acting rev response element. Insert 4 supports expression of rev, vpu and env. The env may have the MHC class II cross reactive epitope altered to eliminate crossreactivity and the V3 loop may be altered to eliminate the possibility of syncytia formation.

In some embodiments, backbone A is used with insert 1. Such constructs optionally contain the SV40 origin of replication. Plasmid pA1ori+ is backbone A with insert 1 and the SV40 origin of replication. Plasmid pA1ori- is backbone A with insert 1 without the SV40 origin of replication. Additionally, either pA1ori+ or pA1ori- may include integrase yielding pA1ori+int+ and pA1ori-int+, respectively. Plasmids pA1ori+, pA1ori-, pA1ori+int+ and pA1ori-int+ may be further modified by functionally deleting the reverse transcriptase (RT) gene yielding pA1ori+RT-, pA1ori-RT-, pA1ori+int+RT- and pA1ori-int+RT-, respectively.

In some embodiments, backbone A is used with insert 2. Such constructs optionally the SV40 origin of replication. Plasmid pA2ori+ is backbone A with insert 2 and the SV40 origin of replication. Plasmid pA2ori- is backbone A with insert 1 without the SV40 origin of replication. Additionally, either pA2ori+ or pA2ori- may include integrase yielding pA2ori+int+ and pA2ori-int+, respectively. Plasmids pA2ori+, pA2ori-, pA2ori+int+ and pA2ori-int+ may be further modified by functionally deleting the reverse transcriptase (RT) gene yielding pA2ori+RT-, pA2ori-RT-, pA2ori+int+RT- and pA2ori-int+RT-, respectively.

In some embodiments, backbone B is used with insert 1. Such constructs optionally the SV40 origin of replication. Plasmid pB1ori+ is backbone B with insert 1 and the SV40 origin of replication. Plasmid pB1ori- is backbone B with insert 1 without the SV40 origin of replication. Additionally, either pB1ori+ or pB1ori- may include integrase yielding pB1ori+int+ and pB1ori-int+, respectively. Plasmids pB1ori+, pB1ori-, pB1ori+int+ and pB1ori-int+ may be further modified by functionally deleting the reverse transcriptase (RT) gene yielding pB1ori+RT-, pB1ori-RT-, pB1ori+int+RT- and pB1ori-int+RT-, respectively.

In some embodiments, backbone B is used with insert 2. Such constructs optionally the SV40 origin of replication. Plasmid pB2ori+ is backbone B with insert 2 and the SV40 origin of replication. Plasmid pB2ori- is backbone B with insert 1 without the SV40 origin of replication. Additionally, either pB2ori+ or pB2ori- may include integrase yielding pB2ori+int+ and pB2ori-int+, respectively. Plasmids

pB2ori+, pB2ori-, pB2ori+int+ and pB2ori-int+ may be further modified by functionally deleting the reverse transcriptase (RT) gene yielding pB2ori+RT-, pB2ori-RT-, pB2ori+int+RT- and pB2ori-int+RT-, respectively.

In some embodiments, backbone A minus rev is used with insert 3. Such constructs optionally the SV40 origin of replication. Plasmid pA/r-3ori+ is backbone A with insert 2 and the SV40 origin of replication. Plasmid pA/r-3ori- is backbone A minus rev with insert 3 without the SV40 origin of replication. Additionally, either pA/r-3ori+ or pA/r-3ori- may include integrase yielding pA/r-3ori+int+ and pA/r-3ori-int+, respectively. Plasmids pA/r-3ori+, pA/r-3ori-, pA/r-3ori+int+ and pA/r-3ori-int+ may be further modified by functionally deleting the reverse transcriptase (RT) gene yielding pA/r-3ori+RT-, pA/r-3ori-RT-, pA/r-3ori+int+RT- and pA/r-3ori-int+RT-, respectively.

In some embodiments, backbone C is used with insert 1. Such constructs optionally the SV40 origin of replication. Plasmid pC1ori+ is backbone C with insert 1 and the SV40 origin of replication. Plasmid pC1ori- is backbone C with insert 1 without the SV40 origin of replication. Additionally, either pC1ori+ or pC1ori- may include integrase yielding pC1ori+int+ and pC1ori-int+, respectively. Plasmids pC1ori+, pC1ori-, pC1ori+int+ and pC1ori-int+ may be further modified by functionally deleting the reverse transcriptase (RT) gene yielding pC1ori+RT-, pC1ori-RT-, pC1ori+int+RT- and pC1ori-int+RT-, respectively.

In some embodiments, backbone C is used with insert 2. Such constructs optionally the SV40 origin of replication. Plasmid pC2ori+ is backbone C with insert 2 and the SV40 origin of replication. Plasmid pC2ori- is backbone C with insert 2 without the SV40 origin of replication. Additionally, either pC2ori+ or pC2ori- may include integrase yielding pC2ori+int+ and pC2ori-int+, respectively. Plasmids pC2ori+, pC2ori-, pC2ori+int+ and pC2ori-int+ may be further modified by functionally deleting the reverse transcriptase (RT) gene yielding pC2ori+RT-, pC2ori-RT-, pC2ori+int+RT- and pC2ori-int+RT-, respectively.

In some embodiments, backbone C is used with insert 3. Such constructs optionally the SV40 origin of replication. Plasmid pC3ori+ is backbone C with insert 3 and the SV40 origin of replication. Plasmid pC3ori- is backbone C with insert 3 without the SV40 origin of replication. Additionally, either pC3ori+ or pC3ori- may include integrase yielding pC3ori+int+ and pC3ori-int+, respectively. Plasmids pC3ori+, pC3ori-, pC3ori+int+ and pC3ori-int+ may be further modified by functionally deleting the reverse transcriptase (RT) gene yielding pC3ori+RT-, pC3ori-RT-, pC3ori+int+RT- and pC3ori-int+RT-, respectively.

In some embodiments, backbone D is used with insert 4. Such constructs optionally the SV40 origin of replication. Plasmid pD4ori+ is backbone D with insert 4 and the SV40 origin of replication. Plasmid pD4ori- is backbone D with insert 4 without the SV40 origin of replication.

#### Example 47

In some embodiments, a single expression unit/single inoculant genetic vaccine is provided which comprises a genetic construct that includes a coding sequence which encodes a peptide that has at least one epitope which is an identical to or substantially similar to epitopes of HIV proteins. The coding sequence is under the regulatory control of the CMV immediate early promoter and the SV40 minor polyadenylation signal.

In some embodiments, a single expression unit/single inoculant genetic vaccine is provided which comprises a

genetic construct that includes a coding sequence which encodes at least one HIV protein or a fragment thereof. The coding sequence is under the regulatory control of the CMV immediate early promoter and the SV40 minor polyadenylation signal. The HIV protein is selected from the group consisting of gag, pol, env and rev. In some embodiments it is preferred that the genetic vaccine is provided which comprises a genetic construct that includes a coding sequence which encodes at least two HIV proteins or a fragments thereof selected from the group consisting of gag, pol, env and rev or fragments thereof. In some embodiments, it is preferred that the genetic vaccine is provided which comprises a genetic construct that includes a coding sequence which encodes at least three HIV proteins or a fragments thereof selected from the group consisting of gag, pol, env and rev or fragments thereof. In some embodiments, it is preferred that the genetic vaccine is provided which comprises a genetic construct that includes a coding sequence which encodes gag, pol, env and rev or fragments thereof.

In some embodiments, a dual expression unit/single inoculant genetic vaccine is provided which comprises a genetic construct that includes two expression units each of which comprises a coding sequence which encodes a peptide that has at least one epitope which is an identical to or substantially similar to epitopes of HIV proteins. The coding sequence is under the regulatory control of the CMV immediate early promoter and the SV40 minor polyadenylation signal. The two expression units are encoded in opposite directions of each other.

In some embodiments, a dual expression unit/single inoculant genetic vaccine is provided which comprises a genetic construct that includes two expression units each of which comprises a coding sequence which encodes at least one HIV protein or a fragment thereof. Each expression unit comprises a coding sequence that is under the regulatory control of the CMV immediate early promoter and the SV40 minor polyadenylation signal. The HIV protein is selected from the group consisting of gag, pol, env and rev. In some embodiments it is preferred that the genetic vaccine is provided which comprises a genetic construct that includes two expression units, at least one of which comprises a coding which encodes at least two HIV proteins or a fragments thereof selected from the group consisting of gag, pol, env and rev or fragments thereof and the other comprises at least one HIV proteins or a fragments thereof selected from the group consisting of gag, pol, env and rev or fragments thereof. In some embodiments, it is preferred that the genetic vaccine is provided which comprises a genetic construct that includes two expression units, at least one of which comprises a coding sequence which encodes at least three HIV proteins or a fragments thereof selected from the group consisting of gag, pol, env and rev or fragments thereof and the other comprises at least one HIV proteins or a fragments thereof selected from the group consisting of gag, pol, env and rev or fragments thereof. In some embodiments, it is preferred that the genetic vaccine is provided which comprises a genetic construct that comprises two expression units and includes a coding sequence which encodes gag, pol, env and rev or fragments thereof.

Table 1

#### Picornavirus Family

##### Genera:

Rhinoviruses: (Medical) responsible for ~50% cases of the common cold.

## 53

Ehteroviruses: (Medical) includes polioviruses, coxsackieviruses, echoviruses, and human enteroviruses such as hepatitis A virus.

Aphoviruses: (Veterinary) these are the foot and mouth disease viruses.

Target antigens: VP1, VP2, VP3, VP4, VPG

Calcivirus Family

Genera:

Norwalk Group of Viruses: (Medical) these viruses are an important causative agent of epidemic gastroenteritis.

Togavirus Family

Genera:

Alphaviruses: (Medical and Veterinary) examples include Senilis viruses, Ross River virus and Eastern & Western Equine\*\*\* encephalitis.

Reovirus: (Medical) Rubella virus.

Flariviridae Family

Examples include: (Medical) dengue, yellow fever, Japanese encephalitis, St. Louis encephalitis and tick borne encephalitis viruses.

Hepatitis C Virus: (Medical) these viruses are not placed in a family yet but are believed to be either a togavirus or a flavivirus. Most similarity is with togavirus family.

Coronavirus

Family: (Medical and Veterinary) Infectious bronchitis virus (poultry) Porcine transmissible gastroenteric virus (pig)

Porcine hemagglutinating encephalomyelitis virus (pig)

Feline infectious peritonitis virus (cats)

Feline enteric coronavirus (cat)

Canine coronavirus (dog)

The human respiratory coronaviruses cause ~40 cases of common cold. EX. 224E, OC43 Note—coronaviruses may cause non-A, B or C hepatitis

Target antigens:

E1—also called M or matrix protein

E2—also called S or Spike protein

E3—also called HE or hemagglutininesterose glycoprotein (not present in all coronaviruses)

N—nucleocapsid

Rhabdovirus Family

Genera: Vesiliovirus

Lyssavirus: (medical and veterinary) rabies

Target antigen: G protein N protein

Filoviridae Family: (Medical)

Hemorrhagic fever viruses such as Marburg and Ebola virus

Paramyxovirus Family:

Genera:

Paramyxovirus: (Medical and Veterinary)

Mumps virus, New Castle disease virus (important pathogen in chickens)

Morbillivirus: (Medical and Veterinary) Measles, canine distemper

Pneumovirus: (Medical and Veterinary)

Respiratory syncytial virus

Orthomyxovirus Family (Medical)

The Influenza virus

Bungavirus Family

Genera:

Bungavirus: (Medical) California encephalitis, LA Crosse

Phlebovirus: (Medical) Rift Valley Fever

Hantavirus: Puremala is a hemahagin fever virus

## 54

Nairvirus (Veterinary) Nairobi sheep disease

Also many unassigned bungaviruses

Arnavirus Family (Medical)

LCM, Lassa fever virus

5 Reovirus Family

Genera:

Reovirus: a possible human pathogen

Rotavirus: acute gastroenteritis in children

10 Orbiviruses: (Medical and Veterinary) Colorado Tick fever, Lebombo (humans) equine encephalosis, blue tongue

Retrovirus Family

Sub-Family:

15 Oncorivirinal: (Veterinary) (Medical) feline leukemia virus, HTLV1 and HTLVII

Lentivirinal: (Medical and Veterinary) HIV, feline immunodeficiency virus, equine infections, anemia virus Spumavirinal

20 Papovavirus Family

Sub-Family:

Polyomaviruses: (Medical) BKU and JCU viruses

Sub-Family:

25 Papillomavirus: (Medical) many viral types associated with cancers or malignant progression of papilloma

Adenovirus (Medical)

EX AD7, ARD., O.B.—cause respiratory disease—some adenoviruses such as 275 cause enteritis

30 Parvovirus Family (Veterinary)

Feline parvovirus: causes feline enteritis

Feline panleucopeniavirus

Canine parvovirus

Porcine parvovirus

35 Herpesvirus Family

Sub-Family:

alphaherpesviridae

Genera:

Simplexvirus (Medical) HSVI, HSVII Varicellovirus: (Medical—Veterinary) pseudorabies—varicella zoster

Sub-Family—betaherpesviridae

Genera:

Cytomegalovirus (Medical)

45 HCMV

Muromegalovirus

Sub-Family:

Gammaherpesviridae

50 Genera:

Lymphocryptovirus (Medical)

EBV—(Burkitts lympho)

Rhadinovirus

55 Poxvirus Family

Sub-Family:

Chordopoxviridae (Medical—Veterinary)

Genera:

Variola (Smallpox)

60 Vaccinia (Cowpox)

Parapoxvirus—Veterinary

Auipoxvirus—Veterinary

Capripoxvirus

Leporipoxvirus

Suipoxvirus

Sub-Family:

55

Entomopoxviridae  
 Hepadnavirus Family  
 Hepatitis B virus  
 Unclassified  
 Hepatitis delta virus

Table 2

## Bacterial pathogens

Pathogenic gram-positive cocci include:

pneumococcal; staphylococcal; and streptococcal.

Pathogenic gram-negative cocci include:

meningococcal; and gonococcal.

Pathogenic enteric gram-negative bacilli include:

enterobacteriaceae; pseudomonas, acinetobacteria and  
 eikenella; melioidosis; salmonella; shigellosis; hemo-  
 philus; chancroid; brucellosis; tularemia; yersinia (pas-  
 teurella); streptobacillus moniliformis and spirillum ;  
 listeria monocytogenes; erysiplothrux rhusiopathiae;  
 diphtheria; cholera; anthrax; donovanosis (granuloma 20  
 inguinale); and bartonellosis.

56

Pathogenic anaerobic bacteria include: tetanus; botulism;  
 other clostridia; tuberculosis; leprosy; and other mycobac-  
 teria. Pathogenic spirochetal diseases include: syphilis; tre-  
 ponematoses: yaws, pinta and endemic syphilis; and lep-  
 tospirosis. Other infections caused by higher pathogen  
 bacteria and pathogenic fungi include: actinomycosis;  
 nocardiosis; cryptococcosis, blastomycosis, histoplasmosis  
 and coccidioidomycosis; candidiasis, aspergillosis, and  
 mucormycosis; sporotrichosis; paracoccidioidomycosis, pet-  
 riellidiosis, torulopsosis, mycetoma and chromomycosis;  
 and dermatophytosis.

Rickettsial infections include rickettsial and rickettsioses.

Examples of mycoplasma and chlamydial infections  
 include: mycoplasma pneumoniae; lymphogranuloma  
 venereum; psittacosis; and perinatal chlamydial infections.

Pathogenic eukaryotes

Pathogenic protozoans and helminths and infections  
 thereby include: amebiasis; malaria; leishmaniasis; trypano-  
 somiasis; toxoplasmosis; pneumocystis carinii; babesiosis;  
 giardiasis; trichinosis; filariasis; schistosomiasis; nema-  
 todes; trematodes or flukes; and cestode (tapeworm) infec-  
 tions.

## SEQUENCE LISTING

## ( 1 ) GENERAL INFORMATION:

( 1 1 ) NUMBER OF SEQUENCES: 34

## ( 2 ) INFORMATION FOR SEQ ID NO:1:

## ( 1 ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 32 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( 1 1 ) MOLECULE TYPE: DNA

( 1 1 ) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGGCOTCTCG AGACAGAGGA GAGCAAGAAA TG

3 2

## ( 2 ) INFORMATION FOR SEQ ID NO:2:

## ( 1 ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 30 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( 1 1 ) MOLECULE TYPE: DNA

( 1 1 ) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TTTCCCTCTA GATAAGCCAT CCAATCACAC

3 0

## ( 2 ) INFORMATION FOR SEQ ID NO:3:

## ( 1 ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 29 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( 1 1 ) MOLECULE TYPE: DNA

( 1 1 ) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAAGGATCCA TGAATAAATA TTTATTGGG

2 9

-continued

## ( 2 ) INFORMATION FOR SEQ ID NO:4:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 30 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ACTGTCGACT TATTTTAAAG CGTTTTTAAG

30

## ( 2 ) INFORMATION FOR SEQ ID NO:5:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 30 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCCAGTTTTG GATCCTTAAA AAAGGCTTGG

30

## ( 2 ) INFORMATION FOR SEQ ID NO:6:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 27 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTGTGAGGGA CAGAATTCCA ATCAGGG

27

## ( 2 ) INFORMATION FOR SEQ ID NO:7:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 24 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAGTGATATC CCGGGAGACT CCTC

24

## ( 2 ) INFORMATION FOR SEQ ID NO:8:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 25 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAATAGAAGA ACTCCTCTAG AATTC

25

## ( 2 ) INFORMATION FOR SEQ ID NO:9:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 27 base pairs
- ( B ) TYPE: nucleic acid

-continued

( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCCTTAGGCG GATCCTATGG CAGGAAG

27

( 2 ) INFORMATION FOR SEQ ID NO:10:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 24 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TAAGATGGGT GGCCATGGTG AATT

24

( 2 ) INFORMATION FOR SEQ ID NO:11:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 32 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGGCGTCTCG AGACAGAGGA GAGCAAGAAA TG

32

( 2 ) INFORMATION FOR SEQ ID NO:12:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 30 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TTTCCCTCTA GATAAGCCAT CCAATCACAC

30

( 2 ) INFORMATION FOR SEQ ID NO:13:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 22 amino acids  
( B ) TYPE: amino acid  
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: peptide

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Cys Asn Thr Arg Lys Arg Ile Arg Ile Glu Arg Gly Pro Gly Arg Ala  
1 5 10 15

Phe Val Thr Ile Gly Lys  
20

( 2 ) INFORMATION FOR SEQ ID NO:14:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 23 amino acids  
( B ) TYPE: amino acid  
( D ) TOPOLOGY: linear

-continued

( i i ) MOLECULE TYPE: peptide

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Tyr Asn Lys Arg Lys Arg Ile His Ile Gln Arg Gly Pro Gly Arg Ala  
 1 5 10 15  
 Phe Tyr Thr Thr Lys Asn Ile Ile Cys  
 20 25

( 2 ) INFORMATION FOR SEQ ID NO:15:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 27 amino acids
- ( B ) TYPE: amino acid
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: peptide

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Cys Arg Ile Lys Gln Phe Ile Asn Met Trp Gln Glu Val Gly Lys Ala  
 1 5 10 15  
 Met Thr Ala Pro Pro Ile Ser Gly Ile Arg Cys  
 20 25

( 2 ) INFORMATION FOR SEQ ID NO:16:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 26 amino acids
- ( B ) TYPE: amino acid
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: peptide

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Arg Ile Leu Ala Val Gln Arg Tyr Ile Lys Asp Gln Gln Leu Leu Gly Ile  
 1 5 10 15  
 Trp Gly Cys Ser Gly Lys Leu Ile Cys  
 20 25

( 2 ) INFORMATION FOR SEQ ID NO:17:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 27 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTGTTTAACT TTTGATCGAT CCATTCC

27

( 2 ) INFORMATION FOR SEQ ID NO:18:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 21 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GATTGTATC GATGATCTGA C

21

( 2 ) INFORMATION FOR SEQ ID NO:19:

( i ) SEQUENCE CHARACTERISTICS:

-continued

( A ) LENGTH: 25 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGTAGTAGCA AAAGAAATAG TTAAG

25

( 2 ) INFORMATION FOR SEQ ID NO:20:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 25 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AATTCTTAAC TATTTCTTTT GCTAC

25

( 2 ) INFORMATION FOR SEQ ID NO:21:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 25 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ATTTGTGCAC TGGTTTCAGC CTGCCATGGC AGGAAGAAGC

40

( 2 ) INFORMATION FOR SEQ ID NO:22:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 29 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ACGACGCGTA TTCTTTAGCT CCTGACTCC

29

( 2 ) INFORMATION FOR SEQ ID NO:23:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 24 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GCTGACGGTA GCGGCCGCAC AATT

24

( 2 ) INFORMATION FOR SEQ ID NO:24:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 22 base pairs  
( B ) TYPE: nucleic acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear



-continued

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GTATTAAGCG GCCGCAATTG TT

2 2

( 2 ) INFORMATION FOR SEQ ID NO:25:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 78 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AAAAAGCTTC GCGGATCCGC GTTGCGGCCG CAACCGGTCA CCGGCGACGC GTCGGTCGA  
CGGTCATGGC TGGGCCCC

6 0

7 8

( 2 ) INFORMATION FOR SEQ ID NO:26:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 29 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CCCAAGCTTA GACATGATAA GATACATTG

2 9

( 2 ) INFORMATION FOR SEQ ID NO:27:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 22 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTAGCAOCTG GATCCAGCT TC

2 2

( 2 ) INFORMATION FOR SEQ ID NO:28:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 24 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GGATTTCTGG GGATCCAAGC TAGT

2 4

( 2 ) INFORMATION FOR SEQ ID NO:29:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 31 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( i i ) MOLECULE TYPE: DNA

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:29:

-continued

TATAGGATCC GCGCAATGAA AGACCCACCC T

31

## ( 2 ) INFORMATION FOR SEQ ID NO:30:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 31 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ATATGGATCC GCAATGAAAG ACCCCCCTG A

31

## ( 2 ) INFORMATION FOR SEQ ID NO:31:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 30 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TAAAGCGGCC GCTCCTATGG CAGGAAGACG

30

## ( 2 ) INFORMATION FOR SEQ ID NO:32:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 34 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:32:

ATTACGCGTC TTATGCTTCT AGCCAGGCAC AATG

34

## ( 2 ) INFORMATION FOR SEQ ID NO:33:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 40 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:33:

ATTACGCGTT TATTACAGAA TGGAAAACAG ATGCCAGGTG

40

## ( 2 ) INFORMATION FOR SEQ ID NO:34:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 32 base pairs
- ( B ) TYPE: nucleic acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

## ( i i ) MOLECULE TYPE: DNA

## ( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ATTACGCGTT ATTGCAGAAT TCTTATTATG GC

32

We claim:

1. A method of immunizing an individual comprising:  
injecting into skeletal muscle tissue of said individual at  
a site on said individual's body, bupivacaine and a  
DNA molecule that comprises a DNA sequence that  
encodes an antigen from a pathogen, said DNA  
sequence operatively linked to regulatory sequences  
which control the expression of said DNA sequence;  
wherein said DNA molecule is taken up by cells in said  
skeletal muscle tissue, said DNA sequence is expressed  
in said cells and an immune response is generated  
against said antigen.
2. The method of claim 1 wherein said pathogen is an  
intracellular pathogen.
3. The method of claim 1 wherein said pathogen is a virus  
selected from the group consisting of: human immunodeficiency  
virus, HIV; human T cell leukemia virus, HTLV;  
influenza virus; hepatitis A virus; hepatitis B virus; hepatitis  
C virus; human papilloma virus, HPV; Herpes simplex 1  
virus, HSV1; Herpes simplex 2 virus, HSV2; Cytomegalo-  
virus, CMV; Epstein-Barr virus, EBR; rhinovirus; and,  
coronavirus.
4. The method of claim 1 wherein said pathogen is HIV  
and said DNA molecule comprises a DNA sequence that  
encodes an HIV antigen.
5. The method of claim 1 wherein at least two non-  
identical DNA molecules are injected into skeletal muscle  
tissue of said individual at different sites on said individual's  
body, said bupivacaine being injected into each of the  
different sites of an individual; said non-identical DNA  
molecules each comprising DNA sequences encoding one or  
more pathogen antigens of the same pathogen.
6. A method of immunizing an individual comprising:  
injecting into skeletal muscle tissue of said individual at  
a site on said individual's body, bupivacaine and a

- DNA molecule that comprises a DNA sequence that  
encodes a hyperproliferative disease-associated protein  
operatively linked to regulatory sequences;
- wherein said DNA molecule is taken up by cells in said  
skeletal muscle tissue, said DNA sequence is expressed  
in said cells, and an immune response is generated  
against said hyperproliferative disease-associated pro-  
tein.
7. The method of claim 6 wherein said DNA molecule  
comprises a DNA sequence encoding a target protein  
selected from the group consisting of: protein products of  
oncogenes myb, myc, fyn, ras, src, neu and trk; protein  
products of translocation gene bcr/abl; P53; variable regions  
of antibodies made by B cell lymphomas; and variable  
regions of T cell receptors of T cell lymphomas.
  8. A method of immunizing an individual comprising:  
injecting into skeletal muscle tissue of said individual,  
bupivacaine and a DNA molecule that comprises a  
DNA sequence that encodes an autoimmune disease-  
associated protein operatively linked to regulatory  
sequences;
  - wherein said DNA molecule is taken up by cells in said  
skeletal muscle tissue, said DNA sequence is expressed  
in said cells and an immune response is generated  
against said autoimmune disease-associated protein.
  9. The method of claim 8 wherein said DNA molecule  
comprises a DNA sequence encoding a target protein  
selected from the group consisting of: variable regions of  
antibodies involved in B cell mediated autoimmune disease;  
and variable regions of T cell receptors involved in T cell  
mediated autoimmune disease.

\* \* \* \* \*



US006962708B1

**(12) United States Patent  
Chambers et al.****(10) Patent No.: US 6,962,708 B1  
(45) Date of Patent: \*Nov. 8, 2005****(54) CHIMERIC FLAVIVIRUS VACCINES****(75) Inventors:** Thomas J. Chambers, St. Louis, MO (US); Thomas P. Monath, Harvard, MA (US); Farshad Gulrahkoo, Melrose, MA (US); Juan Arroyo, S. Weymouth, MA (US)**(73) Assignees:** Acambis, Inc., Cambridge, MA (US); St. Louis University, St. Louis, MO (US)**(\*) Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

**(21) Appl. No.:** 09/121,587**(22) Filed:** Jul. 23, 1998**Related U.S. Application Data****(63)** Continuation-in-part of application No. PCT/US98/03894, filed on Mar. 2, 1998, which is a continuation-in-part of application No. 09/007,664, filed on Jan. 15, 1998, now abandoned, which is a continuation-in-part of application No. 08/807,445, filed on Feb. 28, 1997, now abandoned.**(51) Int. Cl.<sup>7</sup>** ..... C12N 7/01**(52) U.S. Cl.** ..... 424/199.1; 424/218.1; 424/93.1; 435/235.1; 435/236; 435/320.1**(58) Field of Search** ..... 424/199.1, 218.1, 424/93.1; 435/235.1, 236, 320.1**(56) References Cited****U.S. PATENT DOCUMENTS**

6,184,024 B1	2/2001	Lai et al.
6,682,883 B1	1/2004	Monath et al.
6,696,281 B1	2/2004	Chambers et al.
2003/0044773 A1	3/2003	Kleanthous et al.
2003/0129201 A1	7/2003	Monath et al.
2004/0223979 A1	11/2004	Chambers et al.

**FOREIGN PATENT DOCUMENTS**

WO	WO 93/06214	4/1993
WO	98/37911	9/1998

**OTHER PUBLICATIONS**

Rice et al. *The New Biologist* 1(3) 285–296, 1989.\*  
 Chambers et al. *J. Virology* 69(3): 1600–1605, 1995.\*  
 Arroyo et al., “Yellow Fever Vector Live-Virus Vaccines: West Nile Virus Vaccine Development,” *Trends in Molecular Medicine* 7:350–354 (2001).  
 Caufour et al., “Construction, Characterization and Immunogenicity of Recombinant Yellow Fever 17D–Dengue Type 2 Viruses,” *Virus Research* 79:1–14 (2001).  
 Chambers et al., “Mutagenesis of the Yellow Fever Virus NS2B/3 Cleavage Site: Determinants of Cleavage Site Specificity and Effects on Polyprotein Processing and Viral Replication,” *Journal of Virology* 69:1600–1605 (1995).

Chambers et al., “Vaccine Development Against Dengue and Japanese Encephalitis: Report of a World Health Organization Meeting,” *Vaccine* 15:1494–1502 (1997).

Coia et al., “Nucleotide and Complete Amino Acid Sequences of Kunjin Virus: Definitive Gene Order and Characteristics of the Virus-Specified Proteins,” *J. Gen. Virol.* 69:1–21 (1988).

Galler et al., “The Yellow Fever 17D Vaccine Virus: Molecular Basis of Viral Attenuation and its Use as an Expression Vector,” *Brazilian Journal and Biological Research* 30:157–168 (1997).

Galler et al., “Genetic Variability Among Yellow Fever Virus 17D Substrains,” *Vaccine* 16:1–5 (1998).

Guirakhoo et al., “Construction, Safety, and Immunogenicity in Nonhuman Primates of a Chimeric Yellow Fever–Dengue Virus Tetraivalent Vaccine,” *Journal of Virology* 75:7290–7304 (2001).

Mandl et al., “Sequence of the Genes Encoding the Structural Proteins of the Low-Virulence Tick-Borne Flaviviruses Langat TP21 and Yelantsev,” *Virology* 185:891–895 (1991).

Mandl et al., “Complete Genomic Sequence of Powassan Virus: Evaluation of Genetic-Elements in Tick-Borne Versus Mosquito-Borne Flaviviruses,” *Virology* 194:173–184 (1993).

Pletnev et al., “Construction and Characterization of Chimeric Tick-Borne Encephalitis/Dengue Type 4 Viruses,” *Proc. Natl. Acad. Sci. U.S.A.* 89:10532–10536 (1992).

Shiu et al., “Genomic Sequence of the Structural Proteins of Louping Ill Virus: Comparative Analysis with Tick-Borne Encephalitis Virus,” *Virology* 180:411–415 (1991).

Stocks et al., “Signal Peptidase Cleavage at the Flavivirus C–prM Junction: Dependence on the Viral NS2B–3 Protease for Efficient Processing Requires Determinants in C, the Signal Peptide, and prM,” *Journal of Virology* 72:2141–2149 (1998).

Chambers et al., “Yellow Fever/Japanese Encephalitis Chimeric Viruses: Construction and Biological Properties,” *Journal of Virology* 73:3095–3101 (1999).

Duarte dos Santos et al., “Complete nucleotide sequence of yellow fever virus vaccine strains 17DD and 17D–213,” *Virus Research* 35:35–41 (1995).

Bray et al., “Construction of Intertypic Chimeric dengue Viruses by Substitution of Structural Protein Genes,” *Proc. Natl. Acad. Sci. USA*, 88:10342–10346 (1991).

(Continued)

**Primary Examiner**—Lynette R. F. Smith**Assistant Examiner**—Robert A. Zeman**(74) Attorney, Agent, or Firm**—Clark & Elbing LLP**(57) ABSTRACT**

A chimeric live, infectious, attenuated virus containing a yellow fever virus, in which the nucleotide sequence for a prM-E protein is either deleted, truncated, or mutated, so that functional prM-E protein is not expressed, and integrated into the genome of the yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, so that the prM-E protein of the second flavivirus is expressed.

**14 Claims, 19 Drawing Sheets**

OTHER PUBLICATIONS

Venugopal et al., "Towards a New Generation of Flavivirus Vaccines," *Vaccine*, 12:966-975 (1994).

Marchevsky et al., "Phenotypic Analysis of Yellow Fever Virus Derived From Complementary DNA," *American J. Tropical Medicine & Hygiene*, 52:75-80 (1995).

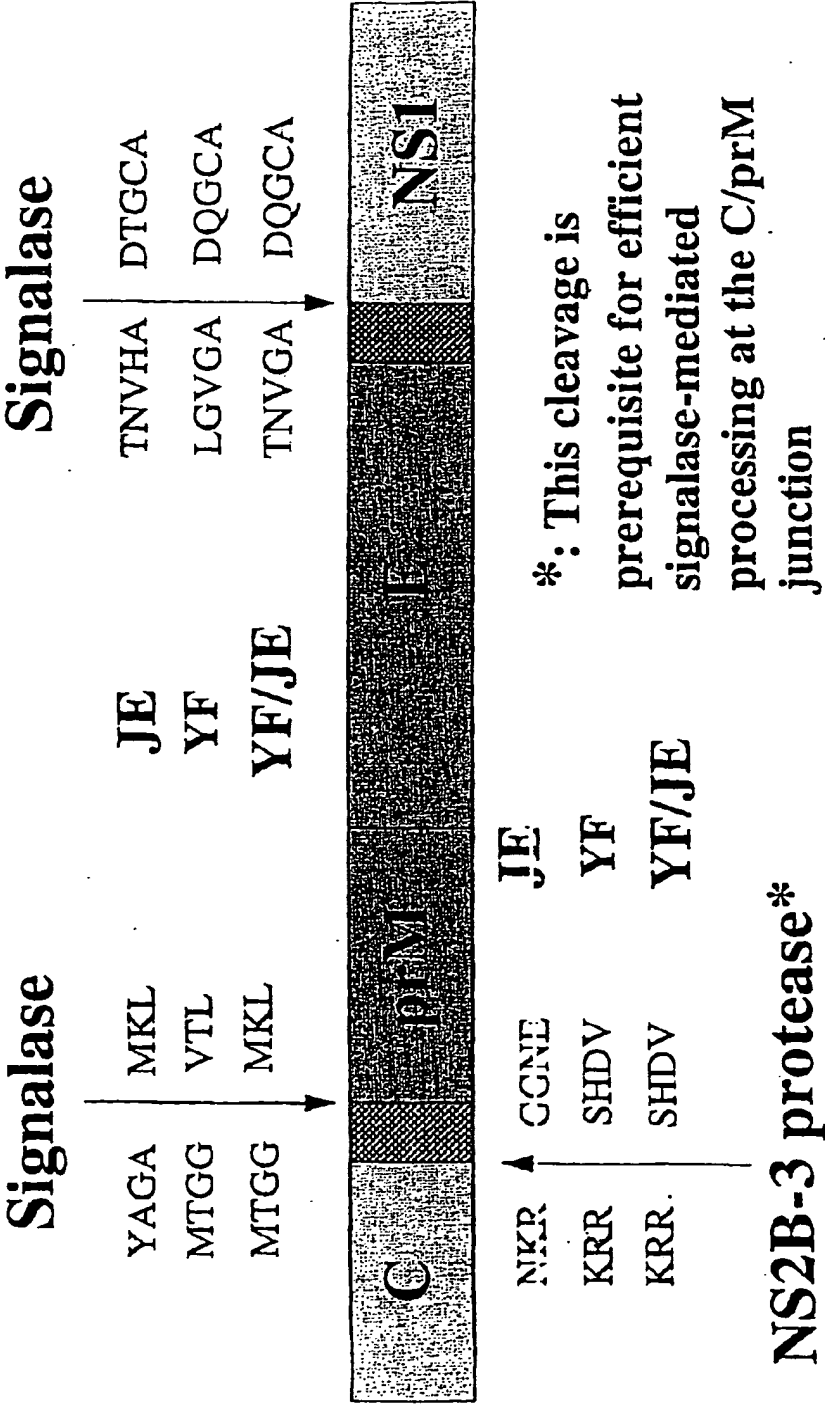
Lai et al., "Evaluation of Molecular Strategies to Develop a Live Dengue Vaccine," *Clinical and Diagnostic Virology* 10:173-179, 1998.

Guirakhoo et al., "Recombinant Chimeric Yellow Fever-Dengue Type 2 Virus is Immunogenic and Protective in Nonhuman Primates," *Journal of Virology* 74:5477-5485, 2000.

Arroyo et al., "Yellow Fever Vector Live-Virus Vaccines: West Nile Virus Vaccine Development," *Trends in Molecular Medicine* 7:350-354, 2001.

\* cited by examiner

**Junction sequences of ChimeriVax™-JE (YF/JE) virus**



\*: This cleavage is prerequisite for efficient signalase-mediated processing at the C/prM junction

09121587.072398  
Fig. 1

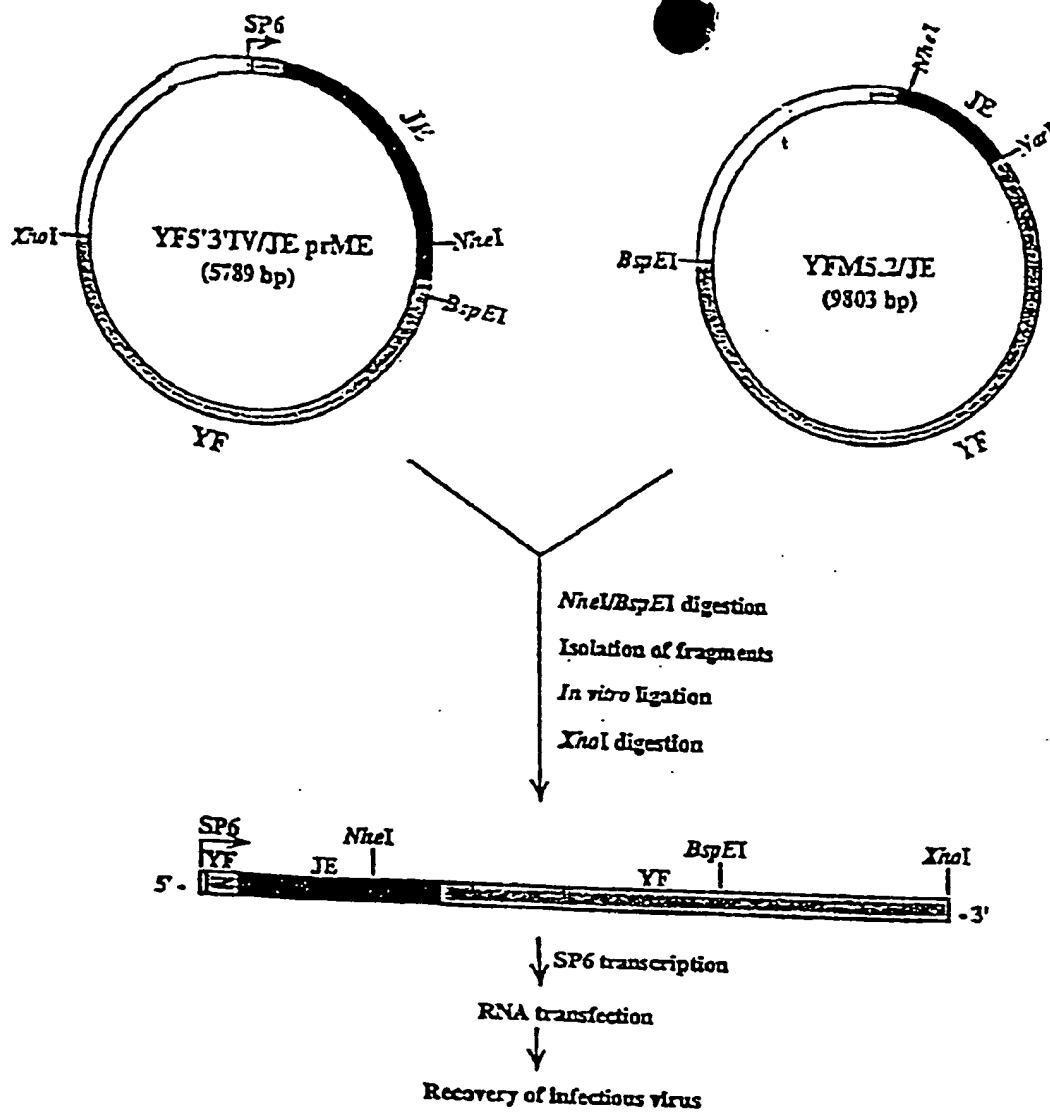


Fig. 2

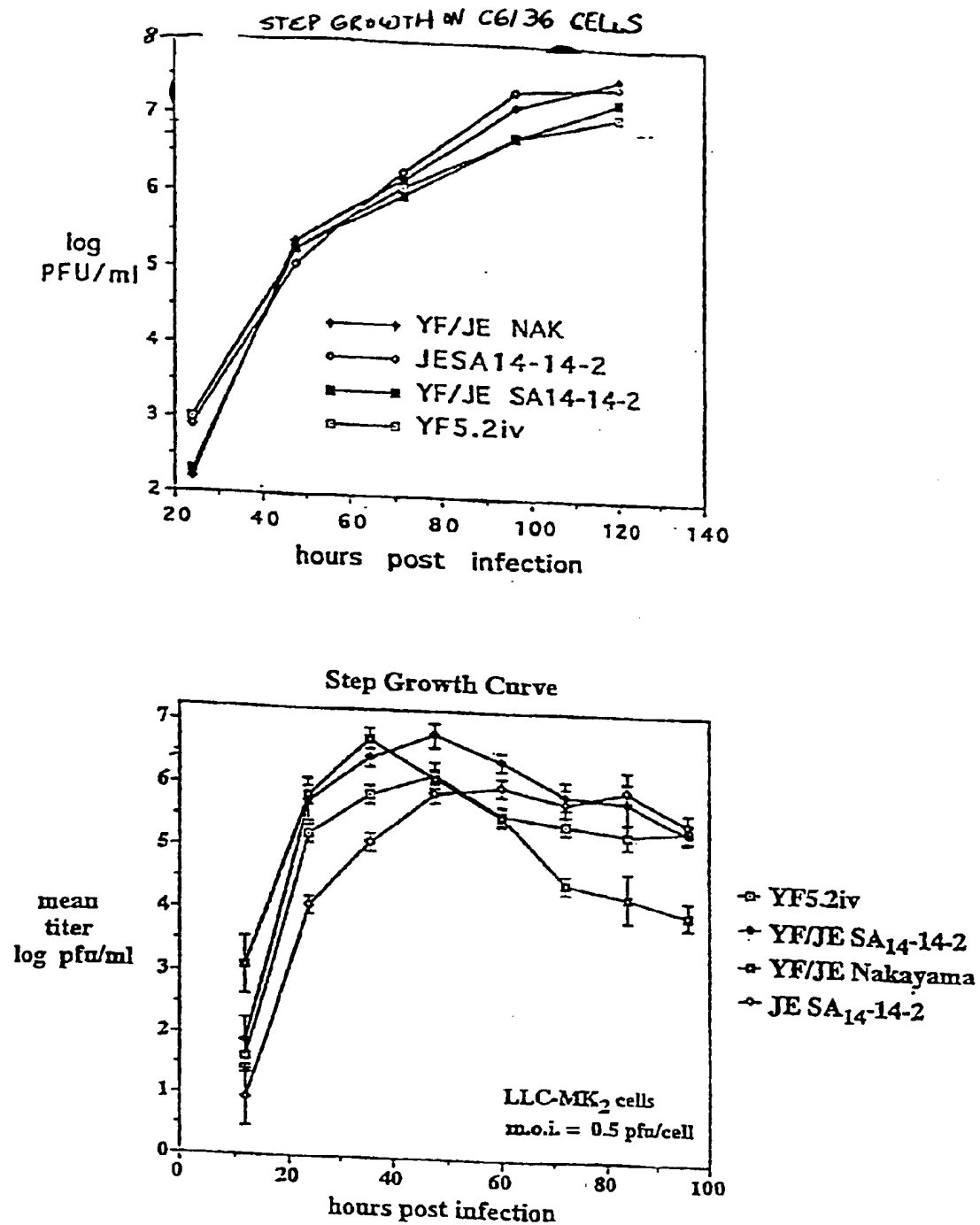


Fig 3



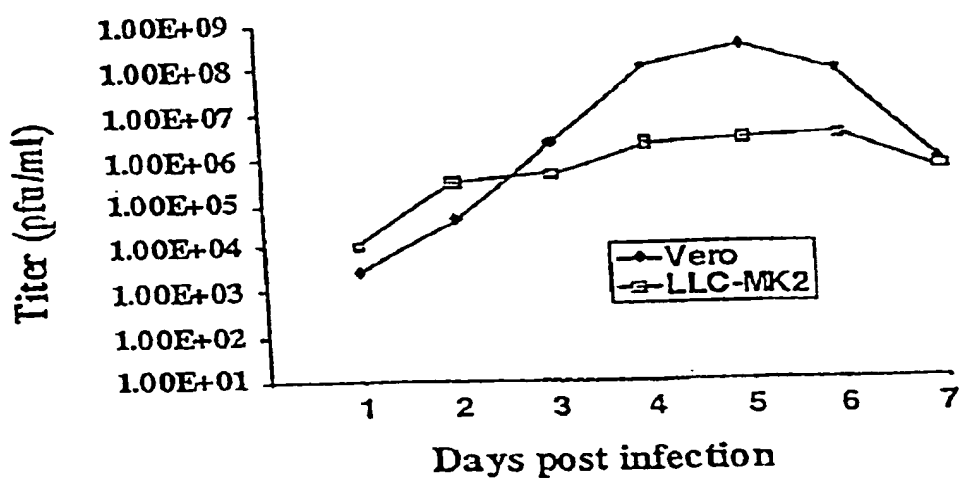
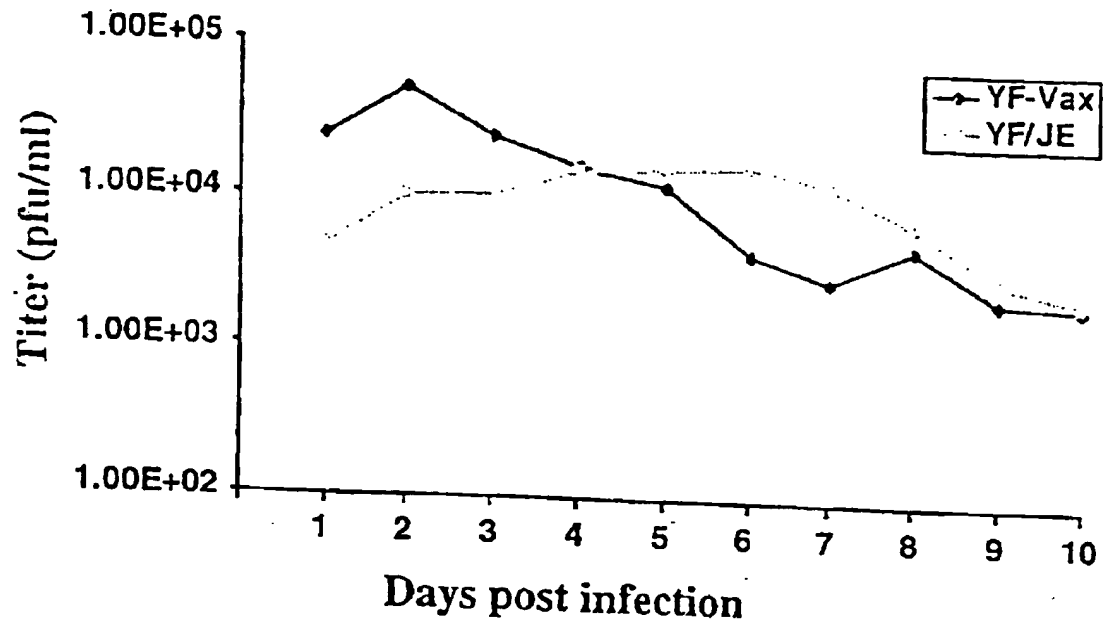


Fig. 4. Growth curves of RMS (YF/JE<sub>SA1+1+2</sub>) in Vero and LLC-MK2 cells.



Growth comparison between RMS and YF-Vax in MRC-5 cells.

Fig. 5

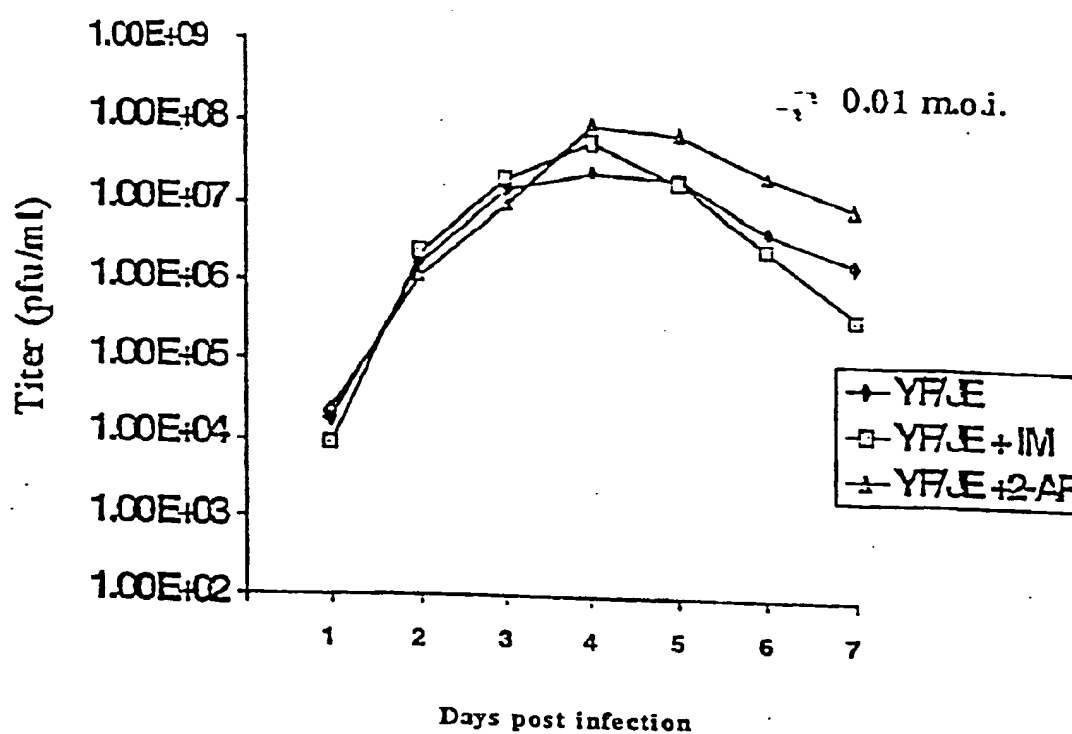


Fig. 6A, Effect of indomethacin (IM) or 2-aminopurine (2-AP) on growth kinetics of YF/JE (0.01 MOI) in FRhL cells

SA14-14-2

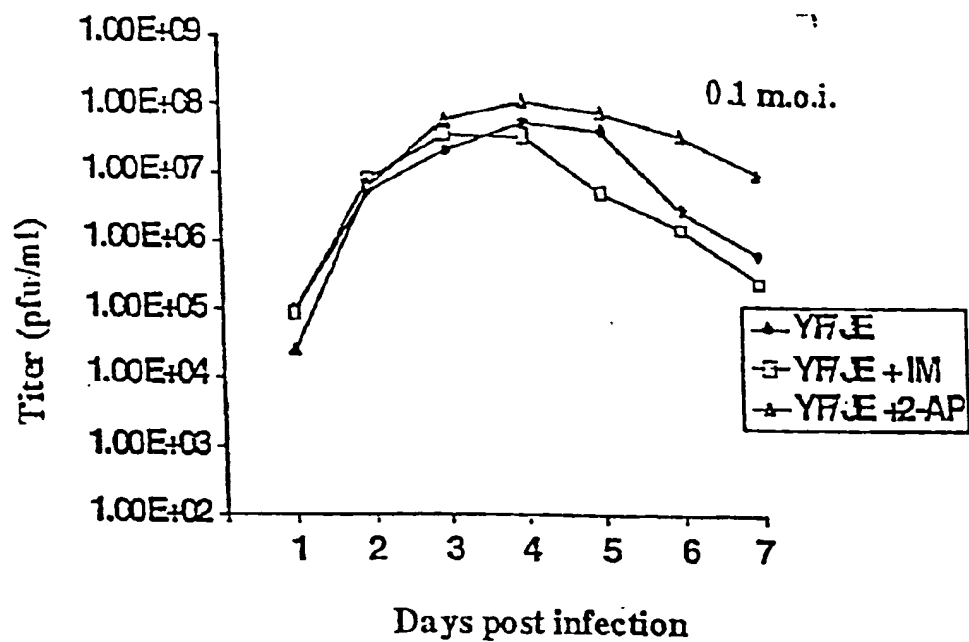
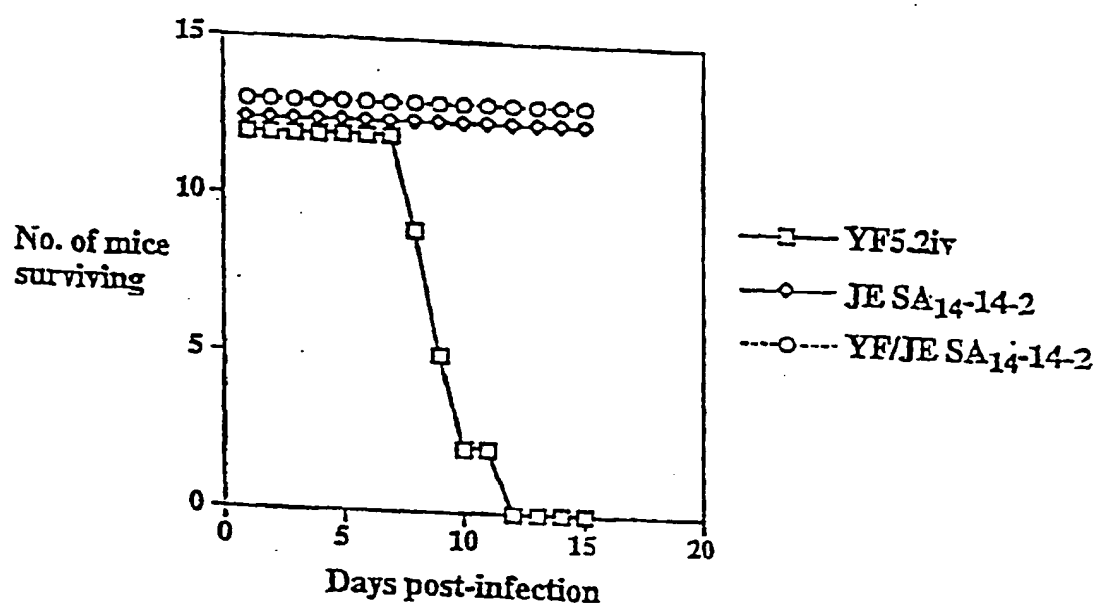


Fig. 6a Effect of indomethacin or 2-aminopurine on growth kinetics of YF/JE<sub>SA1+1+2</sub> (0.1 MOI) in FRhL cells.

### Mouse neurovirulence analysis

MICE: 4 week old ICR males/females  
VIRUS DOSE:  $10^4$  pfu intracerebrally



Virus	Survival	P
YF5.2iv	0/12 (0%)	-
JE SA <sub>14-14-2</sub>	12/12 (100%)	<0.001
YF/JE SA <sub>14-14-2</sub>	13/13 (100%)	<0.001

Fig. 7

Neutralizing antibody response  
to YF/JE SA14-14-2 chimeric vaccine  
(3-week old mice immunized, samples for testing taken at 6 weeks)

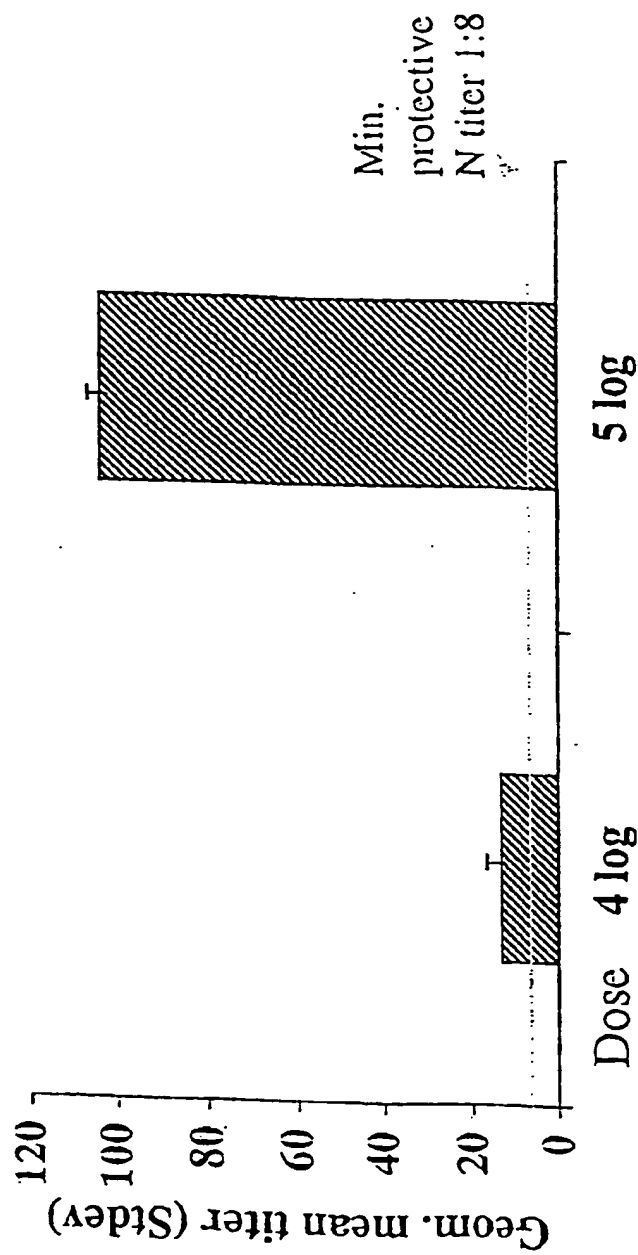


FIG. 8

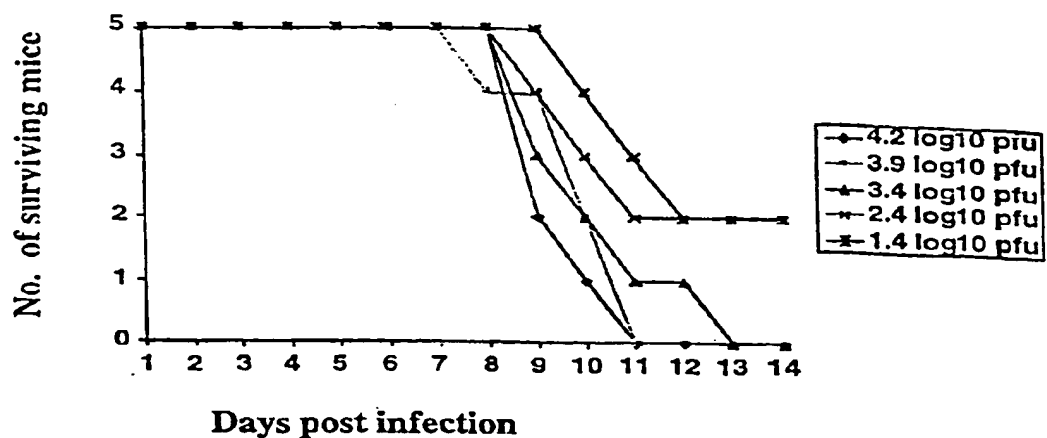


Fig. 9A. Neurovirulence testing of YF-Vax in 4-week old ICR mice by the i.c. route

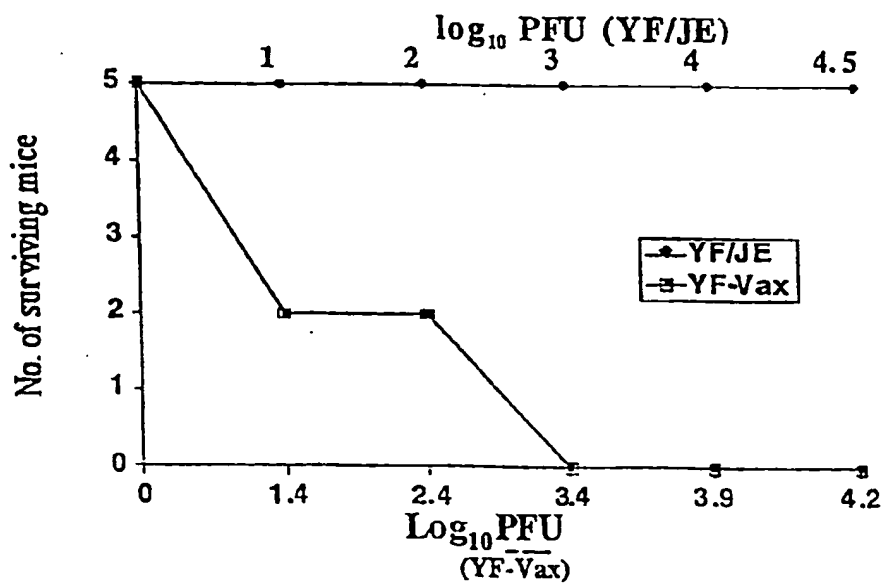


Fig. 9B. Neurovirulence testing of YF/JE<sub>SA14-14-2</sub> in 4-week old ICR mice by i.c. route

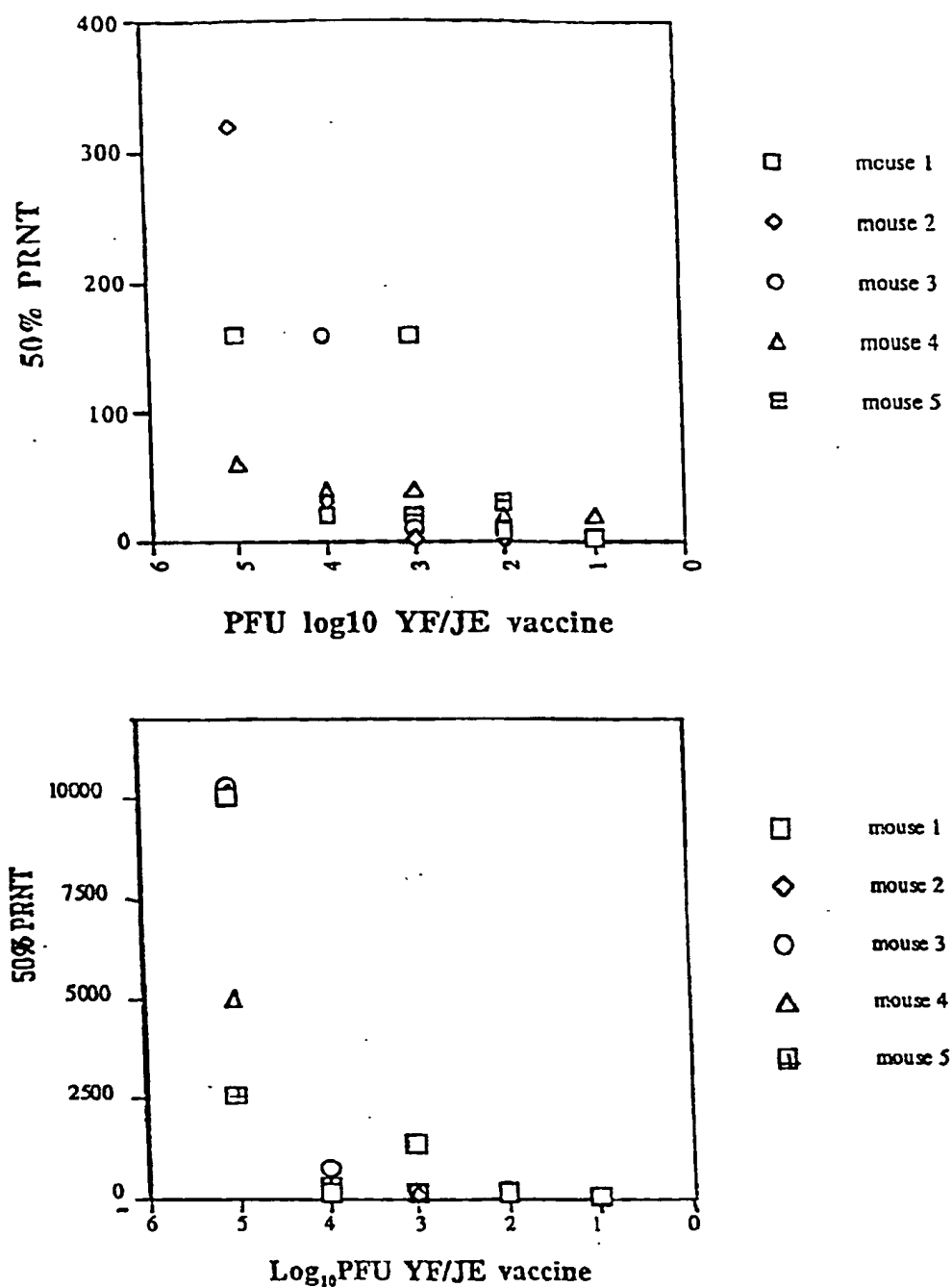


Fig. 10 Neutralizing antibody titers in mice inoculated s.c. with graded doses of YF/JE vaccine. TOP: 3 weeks post immunization and BOTTOM: 8 weeks post immunization



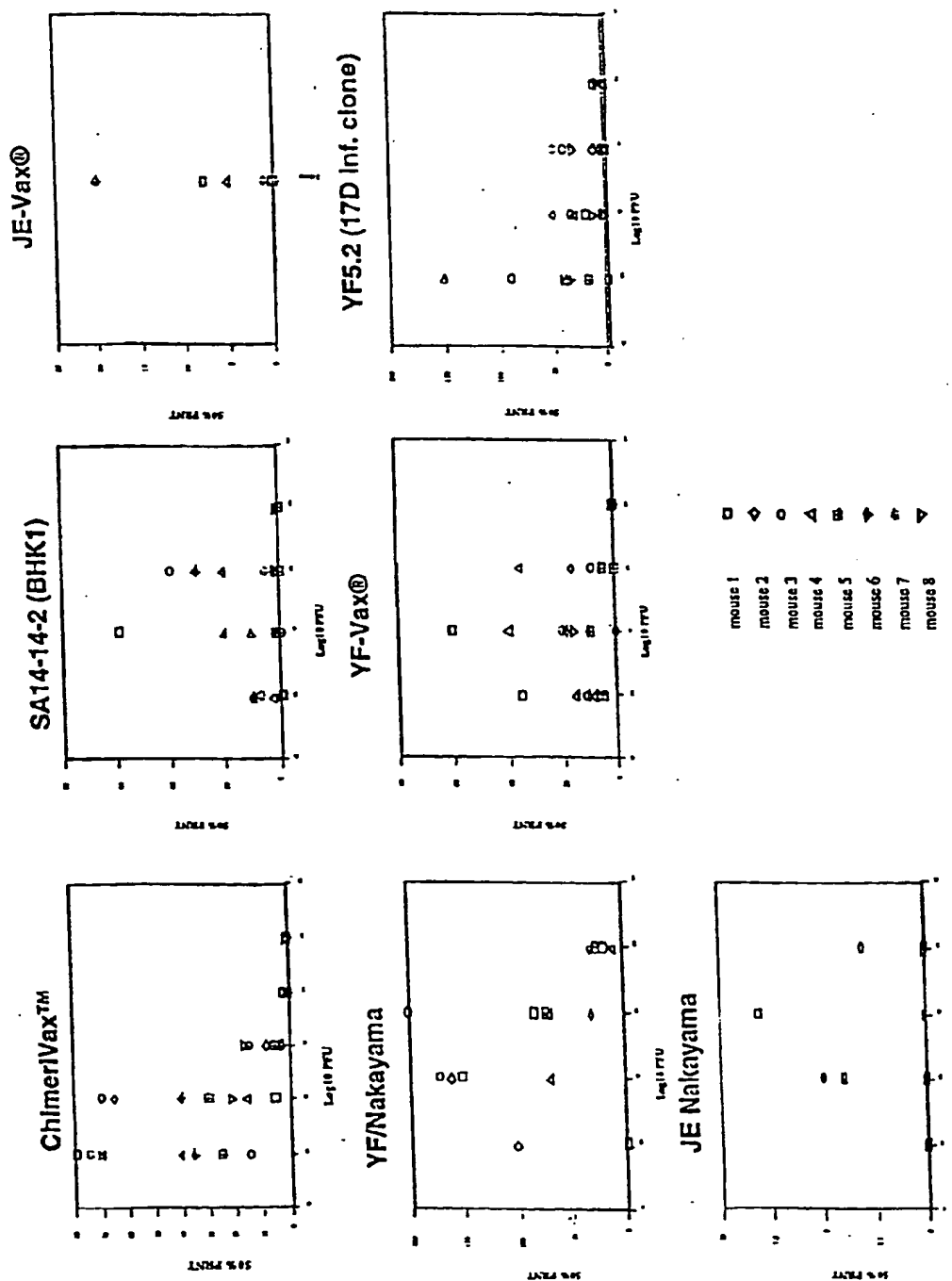


Fig. 1. SEROLOGICAL RESPONSES OF MICE IMMUNIZED WITH A SINGLE DOSE OF LIVE VIRUSES

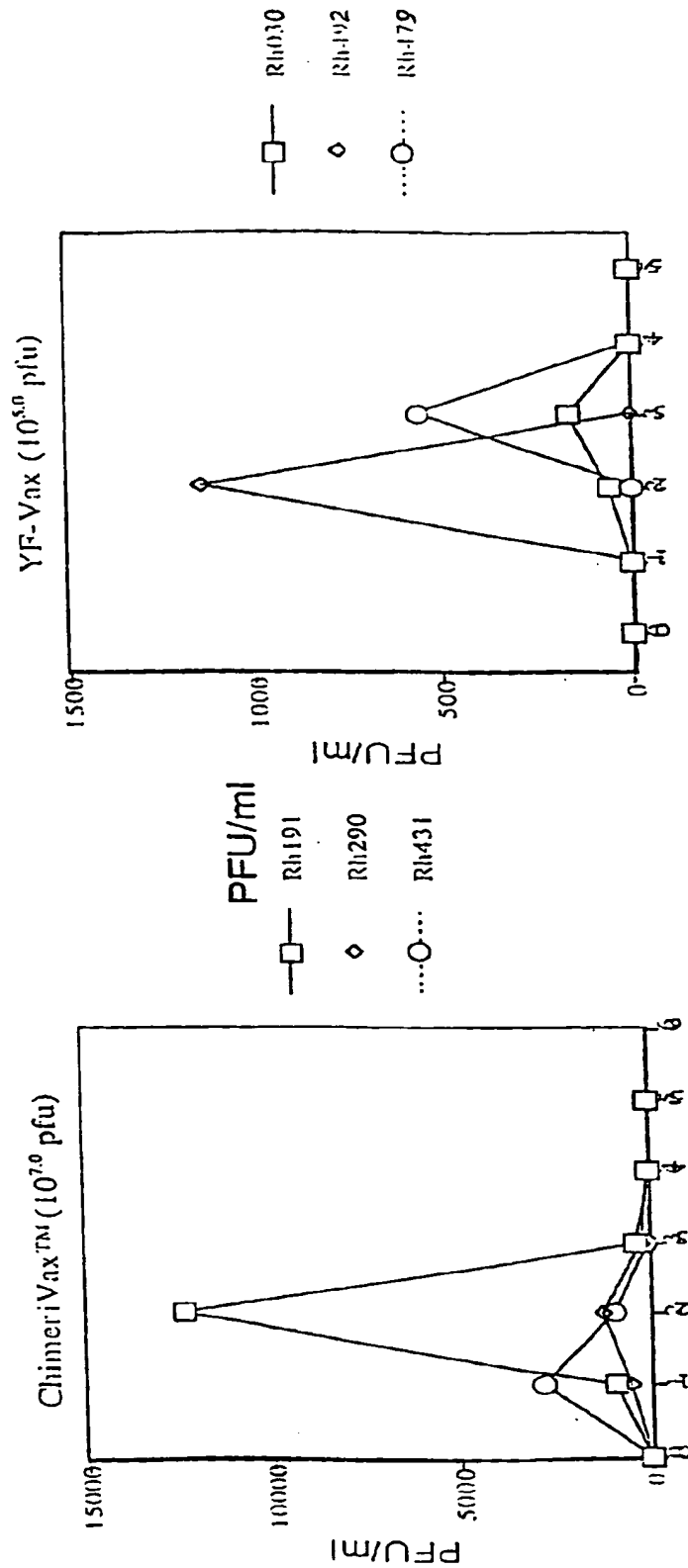


Fig. 12. Viremia and GMT of viremia in 3 rhesus monkeys inoculated with ChimeriVax™ or YF-Vax® by the I.C. route.

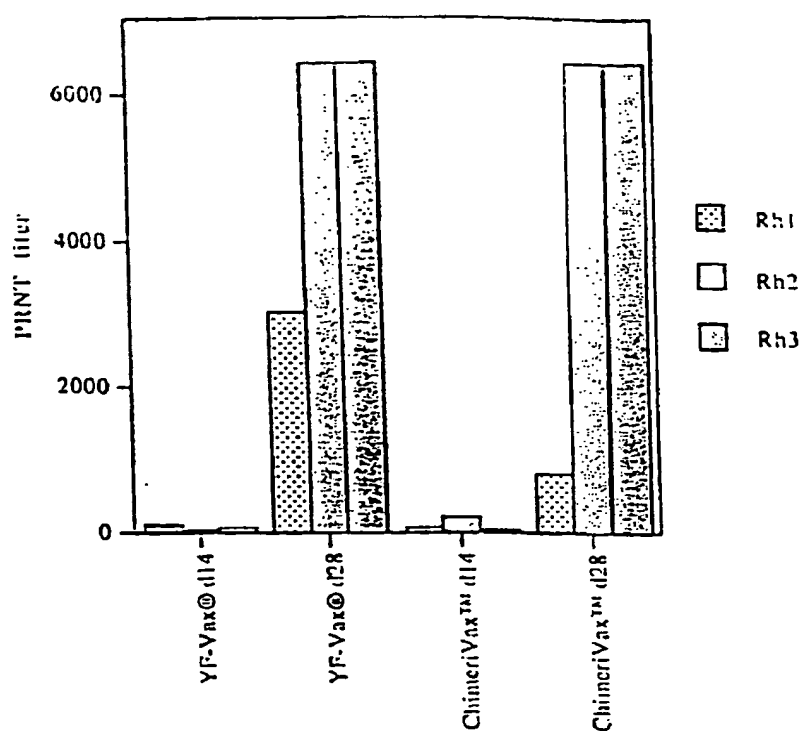


Fig. 13 Neutralizing antibody titers (50%) in rhesus monkeys 2 and 4 weeks post inoculations with a single dose of vaccines by the I.C. route.

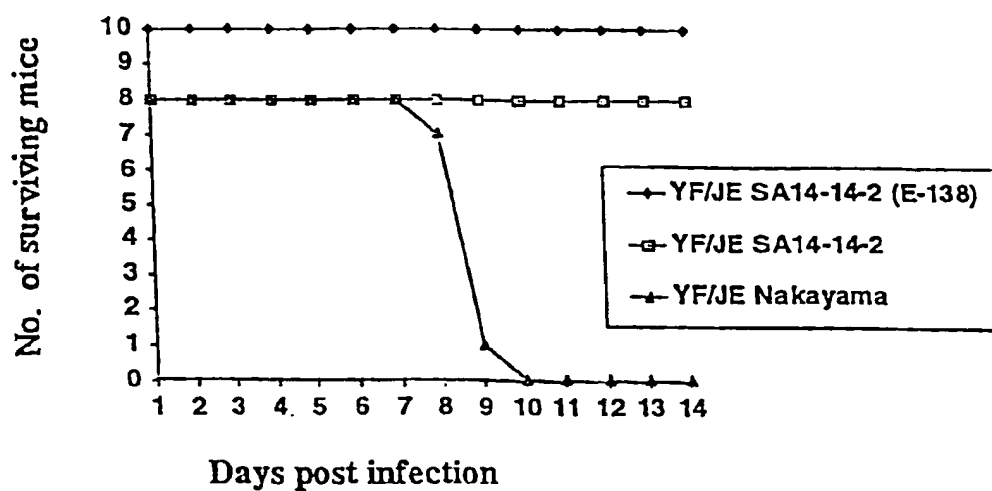


Fig. 17 Mouse neurovirulence testing of YF/JE SA14-14-2 (E-138 K→E) mutant.

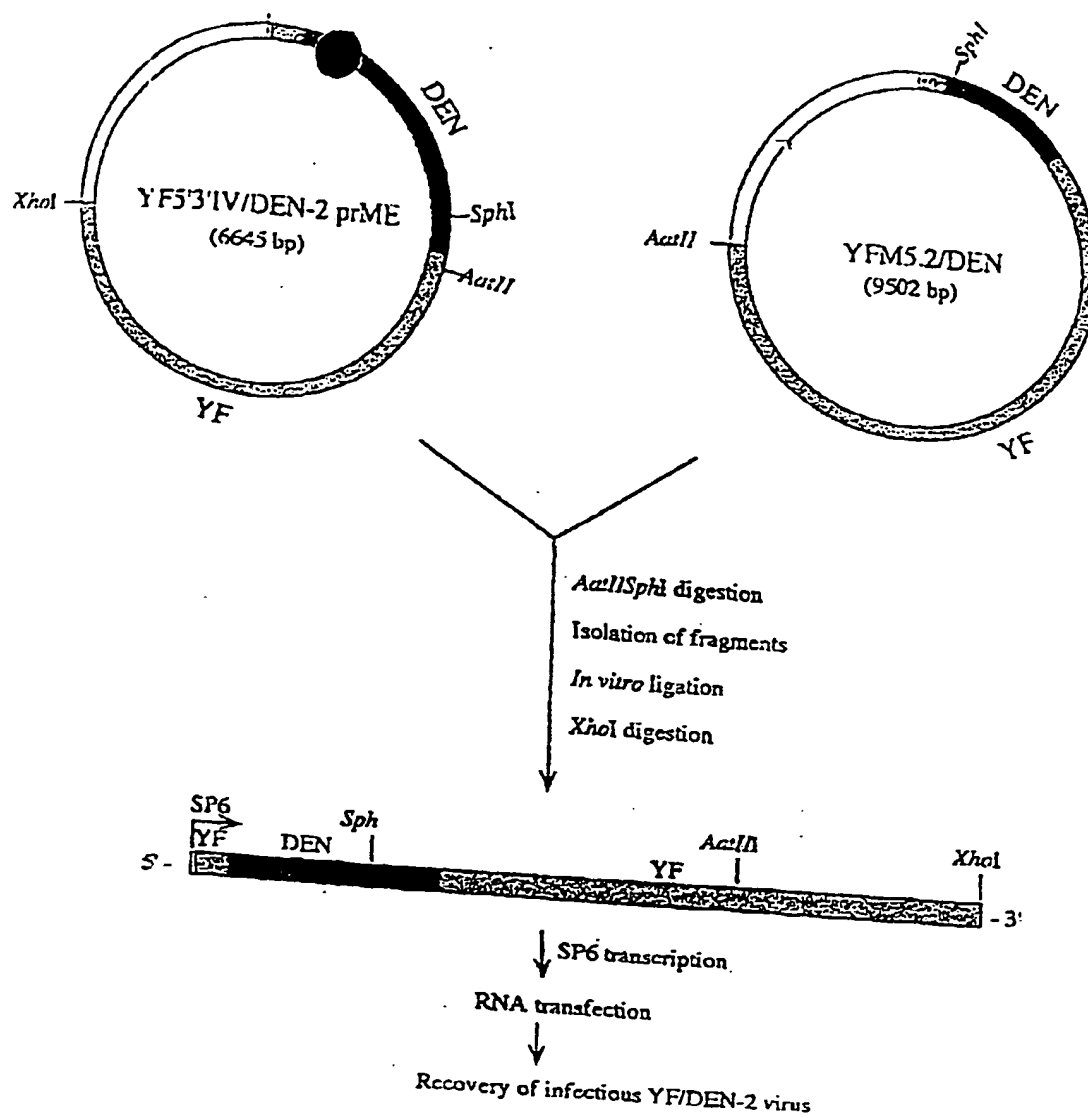


Fig. 15

Structure of modified YF clones expressing  
E/NS1 Intergenic open reading frames

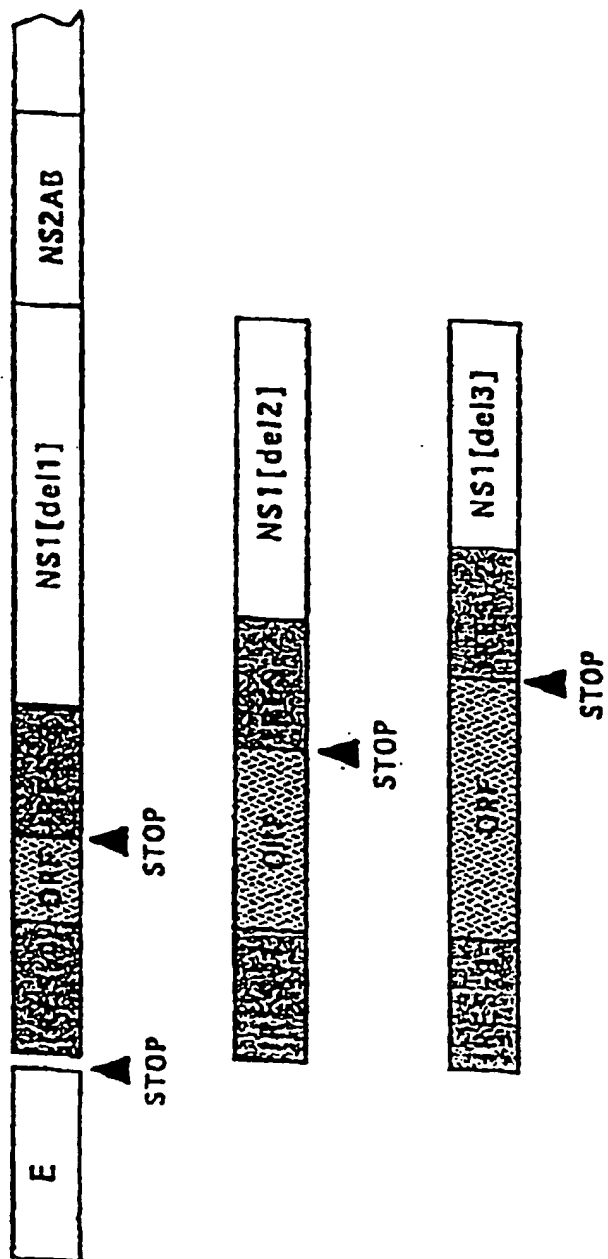


Fig. 16

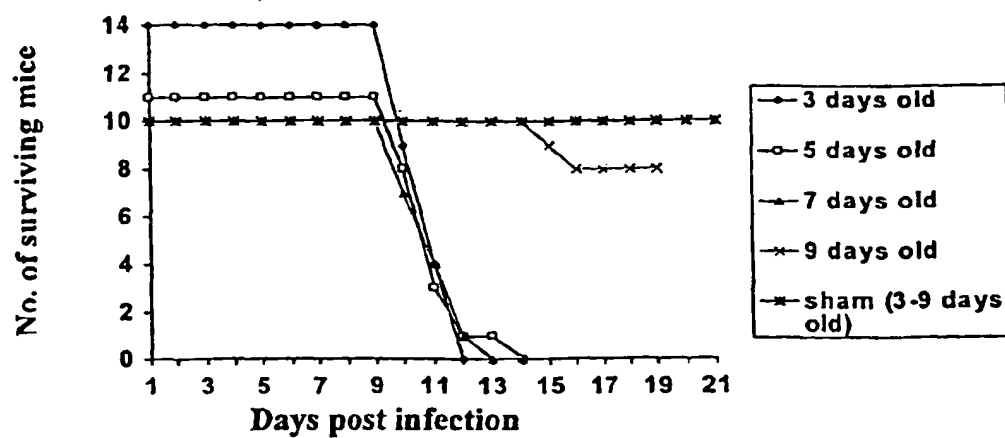


Figure 17. Neurovirulence phenotype of ChimeriVax™-Den2 in outbred (CD-1) suckling mice inoculated by the I.C. route with 10,000 PFU/0.02 ml.

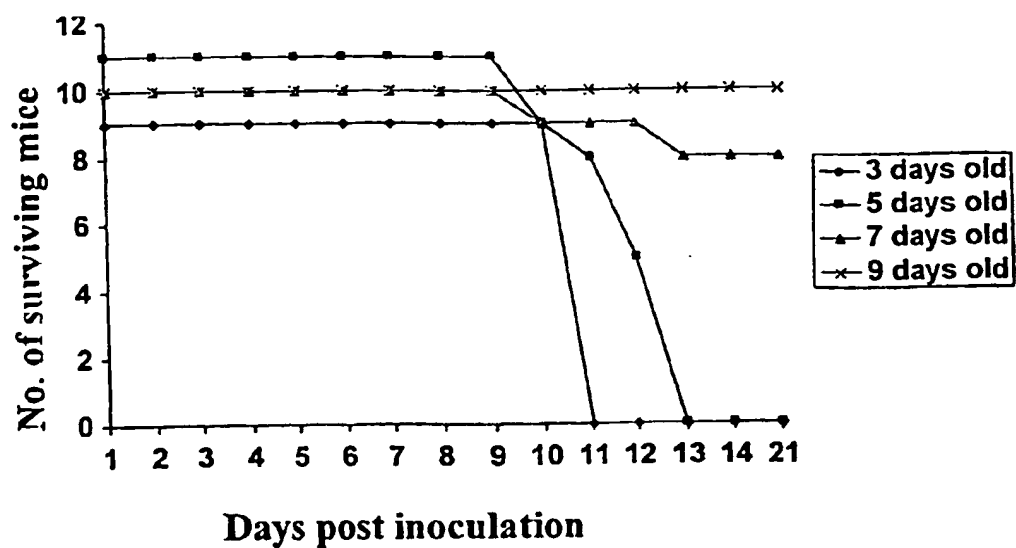


Figure 18. Neurovirulence phenotype of 17D vaccine (YF-Vax®) in outbred (CD-1) suckling mice inoculated by the I.P. route with 1000 PFU/0.02 ml.



## CHIMERIC FLAVIVIRUS VACCINES

This is a continuation-in-part of PCT/US98/03894, filed on Mar. 2, 1998 which is a continuation-in-part of U.S. Ser. No. 09/007,664, filed on Jan. 15, 1998, now abandoned, which is a continuation-in-part of U.S. Ser. No. 08/807,445, filed on Feb. 28, 1997, now abandoned.

## BACKGROUND OF THE INVENTION

This invention relates to infectious, attenuated viruses useful as vaccines against diseases caused by flaviviruses.

Several members of the flavivirus family pose current or potential threats to global public health. For example, Japanese encephalitis is a significant public health problem involving millions of at risk individuals in the Far East. Dengue virus, with an estimated annual incidence of 100 million cases of primary dengue fever and over 450,000 cases of dengue hemorrhagic fever worldwide, has emerged as the single most important arthropod-transmitted human disease.

Other flaviviruses continue to cause endemic diseases of variable nature and have the potential to emerge into new areas as a result of changes in climate, vector populations, and environmental disturbances caused by human activity. These flaviviruses include, for example, St. Louis encephalitis virus, which causes sporadic, but serious, acute disease in the midwest, southeast, and western United States; West Nile virus, which causes febrile illness, occasionally complicated by acute encephalitis, and is widely distributed throughout Africa, the Middle East, the former Soviet Union, and parts of Europe; Murray Valley encephalitis virus, which causes endemic nervous system disease in Australia; and Tick-borne encephalitis virus, which is distributed throughout the former Soviet Union and eastern Europe, where its *Ixodes* tick vector is prevalent and responsible for a serious form of encephalitis in those regions.

Hepatitis C virus (HCV) is another member of the flavivirus family, with a genome organization and replication strategy that are similar, but not identical, to those of the flaviviruses mentioned above. HCV is transmitted mostly by parenteral exposure and congenital infection, is associated with chronic hepatitis that can progress to cirrhosis and hepatocellular carcinoma, and is a leading cause of liver disease requiring orthotopic transplantation in the United States.

The Flaviviridae family is distinct from the alphaviruses (e.g., WEE, VEE, EEE, SFV, etc.) and currently contains three genera, the flaviviruses, the pestiviruses, and the hepatitis C viruses. Fully processed mature virions of flaviviruses contain three structural proteins, envelope (E), capsid (C), and membrane (M), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). Immature flavivirions found in infected cells contain pre-membrane (prM) protein, which is the precursor to the M protein.

After binding of virions to host cell receptors, the E protein undergoes an irreversible conformational change upon exposure to the acidic pH of endosomes, causing fusion between the envelope bilayers of the virions and endocytic vesicles, thus releasing the viral genome into the host cytosol. PrM-containing tick-borne encephalitis (TBE) viruses are fusion-incompetent, indicating that proteolytic processing of prM is necessary for the generation of fusion-competent and fully infectious virions (Guirakhoo et al., 1. Gen. Virol. 72(Pt. 2):333-338, 1991). Using ammonium chloride late in the virus replication cycle, prM-containing

Murray Valley encephalitis (MVE) viruses were produced and shown to be fusion incompetent. By using sequence-specific peptides and monoclonal antibodies, it was demonstrated that prM interacts with amino acids 200-327 of the E protein. This interaction is necessary to protect the E protein from the irreversible conformational changes caused by maturation in the acidic vesicles of the exocytic pathway (Guirakhoo et al., Virology 191:921-931, 1992).

The cleavage of prM to M protein occurs shortly before release of virions by a furin-like cellular protease (Stadler et al., J. Virol. 71:8475-8481, 1997), which is necessary to activate hemagglutinating activity, fusogenic activity, and infectivity of virions. The M protein is cleaved from its precursor protein (prM) after the consensus sequence R-X-R/K-R (X is variable), and incorporated into the virus lipid envelope together with the E protein.

Cleavage sequences have been conserved not only within flaviviruses, but also within proteins of other, unrelated viruses, such as PE2 of murine coronaviruses, PE2 of alphaviruses, HA of influenza viruses, and p160 of retroviruses. Cleavage of the precursor protein is essential for virus infectivity, but not particle formation. It was shown that, in case of a TBE-dengue 4 chimera, a change in the prM cleavage site resulted in decreased neurovirulence of this chimera (Pletnev et al., J. Virol. 67:4956-4963, 1993), consistent with the previous observation that efficient processing of the prM is necessary for full infectivity (Guirakhoo et al., 1991, supra; Guirakhoo et al., 1992, supra; Heinz et al., Virology 198:109-117, 1994). Antibodies to prM protein can mediate protective immunity, apparently due to neutralization of released virions that contain some uncleaved prM. The proteolytic cleavage site of the PE2 of VEE (4 amino acids) was deleted by site-directed mutagenesis of the infectious clone (Smith et al., ASTMH meeting, Dec. 7-11, 1997). Deletion mutants replicated with high efficiency and PE2 proteins were incorporated into particles. This mutant was evaluated in lethal mouse and hamster models and shown to be attenuated; in non-human primates it caused 100% seroconversion and protected all immunized monkeys from a lethal challenge.

## SUMMARY OF THE INVENTION

The invention features chimeric, live, infectious, attenuated viruses that are each composed of:

- a first yellow fever virus (e.g., strain 17D), representing a live, attenuated vaccine virus, in which the nucleotide sequence encoding the prM-E protein is either deleted, truncated, or mutated so that the functional prM-E protein of the first flavivirus is not expressed, and
- integrated into the genome of the first flavivirus, a nucleotide sequence encoding the viral envelope (prM-E) protein of a second, different flavivirus, so that the prM-E protein of the second flavivirus is expressed from the altered genome of the first flavivirus.

The chimeric virus is thus composed of the genes and gene products responsible for intracellular replication belonging to the first flavivirus and the genes and gene products of the envelope of the second flavivirus. Since the viral envelope contains antigenic determinants responsible for inducing neutralizing antibodies, the result of infection with the chimeric virus is that such antibodies are generated against the second flavivirus.

A preferred live virus for use as the first yellow fever virus in the chimeric viruses of the invention is YF 17D, which has been used for human immunization for over 50 years.

YF 17D vaccine is described in a number of publications, including publications by Smithburn et al. ("Yellow Fever Vaccination," World Health Org., p. 238, 1956), and Freestone (in Plotkin et al., (Eds.), *Vaccines*, 2<sup>nd</sup> edition, W. B. Saunders, Philadelphia, 1995). In addition, the yellow fever virus has been studied at the genetic level (Rice et al., *Science* 229:726-733, 1985) and information correlating genotype and phenotype has been established (Marchevsky et al., *Am. J. Trop. Med. Hyg.* 52:75-80, 1995). Specific examples of yellow fever substrains that can be used in the invention include, for example, YF 17DD (GenBank Accession No. U17066), YF 17D-213 (GenBank Accession No. U17067), YF 17D-204 France (X15067, X15062), and YF-17D-204, 234 US (Rice et al., *New Biologist* 1:285-296, 1989; C 03700, K 02749). Yellow Fever virus strains are also described by Galler et al., *Vaccine* 16 (9/10):1024-28, 1998.

Preferred flaviviruses for use as the second flavivirus in the chimeric viruses of the invention, and thus sources of immunizing antigen, include Japanese Encephalitis (JE, e.g., JE SA<sub>14</sub>-14-2), Dengue (DEN, e.g., any of Dengue types 14; for example, Dengue-2 strain PUO-218) (Gruenberg et al., *J. Gen. Virol.* 67:1391-1398, 1988) (sequence appendix 1 (SEQ ID NO: 1); nucleotide sequence of Dengue-2 insert; Pr-M: nucleotides 1-273; M: nucleotides 274-498; E: nucleotides 499-1983) (sequence appendix 1 (SEQ ID NO:2); amino acid sequence of Dengue-2 insert; Pr-M: amino acids 1-91; M: amino acids 92-166; E: amino acids 167-661), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-borne Encephalitis (TBE) (i.e., Central European Encephalitis (CEE) and Russian Spring-Summer Encephalitis (RSSE) viruses), and Hepatitis C(HCV) viruses. Additional flaviviruses for use as the second flavivirus include Kunjin virus, Powassan virus, Kyasanur Forest Disease virus, and Omsk Hemorrhagic Fever virus. As is discussed further below, the second flavivirus sequences can be provided from two different second flaviviruses, such as two Dengue strains.

It is preferable to use attenuated inserts, for example, in the case of inserts from neurotropic viruses, such as JE, MVE, SLE, CEE, and RSSE. In the case of non-neurotropic viruses, such as dengue viruses, it may be preferable to use unmodified inserts, from unattenuated strains. Maintenance of native sequences in such inserts can lead to enhanced immunogenicity of the proteins encoded by the inserts, leading to a more effective vaccine.

In a preferred chimeric virus of the invention, the prM-E protein coding sequence of the second flavivirus is substituted for the prM-E protein coding sequence of the live yellow fever virus. Also, as is described further below, the prM portion of the protein can contain a mutation or mutations that prevent cleavage to generate mature membrane protein.

Also included in the invention are methods of preventing or treating flavivirus infection in a mammal, such as a human, by administering a chimeric flavivirus of the invention to the mammal; use of the chimeric flaviviruses of the invention in the preparation of medicaments for preventing or treating flavivirus infection; nucleic acid molecules encoding the chimeric flaviviruses of the invention; and methods of manufacturing the chimeric flaviviruses of the invention.

The invention provides several advantages. For example, because they are live and replicating, the chimeric viruses of the invention can be used to produce long-lasting protective immunity. Also, because the viruses have the replication genes of an attenuated virus (e.g., Yellow Fever 17D), the

resulting chimeric virus is attenuated to a degree that renders it safe for use in humans.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of the C, prM, E, and NS 1 regions and junction sequences of a YF/JE chimera of the invention. The amino acid sequences flanking cleavage sites at the junctions are indicated for JE, YF, and the YF/JE chimera (SEQ ID NOs:3-11).

FIG. 2 is a schematic representation of genetic manipulation steps that were carried out to construct a Yellow-Fever/Japanese Encephalitis (YF/JE) chimeric virus of the invention.

FIG. 3 is a set of growth curves for chimeric YF/JE viruses of the invention in cell cultures acceptable for preparation of a human vaccine.

FIG. 4 is a growth curve of RMS (Research Master Seed, YF/JE SA<sub>14</sub>-14-2) in Vero and LLC-MK2 cells.

FIG. 5 is a graph showing a growth comparison between RMS (YF/JE SA<sub>14</sub>-14-2) and YF-VAX® (Yellow Fever 17D vaccine) in MRC-5 cells.

FIG. 6A is a graph showing the effects of indomethacin (IM) or 2-aminopurine (2-AP) on growth kinetics of YF/JE SA<sub>14</sub>-14-2 (0.01 MOI) in FRhL cells.

FIG. 6B is a graph showing the effects of indomethacin (IM) or 2-aminopurine (2-AP) on growth kinetics of YF/JE SA<sub>14</sub>-14-2 (0.1 MOI) in FRhL cells.

FIG. 7 is a graph and a table showing the results of a mouse neurovirulence analysis carried out with a YF/JE chimeric virus of the invention.

FIG. 8 is a graph showing the neutralizing antibody response of mice immunized with a YF/JE SA<sub>14</sub>-14-2 chimeric vaccine of the invention. Three week old mice were immunized, and samples for testing were taken at 6 weeks.

FIG. 9A is a graph showing the results of neurovirulence testing of YF-VAX® (Yellow Fever 17D vaccine) in 4 week old ICR mice by the i.c. route.

FIG. 9B is a graph showing the results of neurovirulence testing of YF/JE SA<sub>14</sub>-14-2 in 4 week old ICR mice by the i.c. route.

FIG. 10 is a set of graphs showing the results of PRNT analysis of neutralizing antibody titers in mice inoculated s.c. with graded doses of YF/JE vaccine. The results in the top graph are 3 weeks post immunization, and the results in the bottom graph are 8 weeks post immunization.

FIG. 11 is a series of graphs showing the serological responses of mice immunized with a single dose of the live viruses indicated in the figure.

FIG. 12 is a set of graphs showing viremia and GMT of viremia in 3 rhesus monkeys inoculated with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) or YF-VAX® (Yellow Fever 17D vaccine) by the i.c. route.

FIG. 13 is a graph showing the PRNT neutralizing antibody titers (50%) in rhesus monkeys 2 and 4 weeks post inoculation with a single dose of YF-VAX® (Yellow Fever 17 D vaccine) or CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) vaccines by the i.c. route.

FIG. 14 is a graph showing the results of neurovirulence testing of YF/JE SA<sub>14</sub>-14-2 (E-138 K→mutant).

FIG. 15 is a schematic representation of a two plasmid system for generating chimeric YF/DEN-2 virus. The strategy is essentially as described for the YF/JE chimeric virus.

FIG. 16 is a schematic representation of the structure of modified YF clones designed to delete portions of the NS1 protein and/or express foreign proteins under control of an internal ribosome entry site (IRES). The figure shows only the E/NS1 region of the viral genome. A translational stop codon is introduced at the carboxyl terminus of the envelope (E) protein. Downstream translation is initiated within an intergenic open reading frame (ORF) by IRES-1, driving expression of foreign proteins (e.g., HCV proteins E1 and/or E2). The second IRES (IRES-2) controls translational initiation of the YF nonstructural region, in which nested, truncated NS1 proteins (e.g., NS1del-1, NS1del-2, or NS1del-3) are expressed. The size of the NS1 deletion is inversely proportional to that of the ORF linked to IRES-1.

FIG. 17 is a graph showing the neurovirulence phenotype of CHIMERIVAX™-DEN2 (chimeric flavivirus vaccine comprising Dengue 2 virus prM and E proteins) in outbred (CD-1) suckling mice inoculated by the I.C. route with 10,000 PFU/0.02 ml.

FIG. 18 is a graph showing the neurovirulence phenotype of 17 D vaccine (YF-VAX® (Yellow Fever 17D vaccine) in outbred (CD-1) suckling mice inoculated by the I.P. route with 1000 PFU/0.02 ml.

#### DETAILED DESCRIPTION

The invention provides chimeric flaviviruses that can be used in vaccination methods against flavivirus infection. Construction and analysis of chimeric flaviviruses of the invention, such as chimeras of yellow fever virus and Japanese Encephalitis (JE), Dengue types 14 (DEN 1-4), Murray Valley Encephalitis (MVE), St. Louis Encephalitis (SLE), West Nile (WN), Tick-borne Encephalitis (TBE), and Hepatitis C(HCV) viruses are described as follows.

Flavivirus proteins are produced by translation of a single, long open reading frame (encoding, i.e., the structural proteins, capsid (C), pre-membrane (pr-M), and envelope (E), as well as non-structural proteins (e.g., NS1)) and a complex series of post-translational proteolytic cleavages. The chimeric flaviviruses of the invention, as is discussed above, include those in which the pr-M and E proteins of one flavivirus (yellow fever virus) have been replaced by the pr-M and E proteins of another flavivirus. Thus, creation of these chimeric flaviviruses involves the generation of novel junctions between the capsid and pre-membrane protein, and the envelope protein and the non-structural region (NS1), of two different flaviviruses.

Cleavage between C/pr-M and E/NS1 occurs during the natural proteolytic processing of flavivirus proteins, and requires the presence of signal peptidase, or signalase, recognition sequences flanking the junctions of the cleavage sites. Cleavage at the signalase recognition sites is mediated by host cell signalase. Cleavage by viral NS2B-3 protease in the carboxyl one third of the C protein is required for separation of the cytoplasmic and membrane-anchored portions of the C protein and influences efficiency of cleavage by signalase at the C/pr-M site (Stocks et al., J. Virol. 72(3):2141-2149, 1998; also see FIG. 1).

In the chimeric flaviviruses of the invention, it is preferred that the signalase recognition sites, NS2B-3 protease recognition site, and cleavage sites of the viruses making up the chimeras are substantially maintained, so that proper cleavage between the C and pr-M and E and NS1 proteins can efficiently take place. For example, as is shown in FIG. 1,

with respect to a YF/JE chimera of the invention, the YF NS2 B-3 protease recognition site is maintained in the chimera. Thus, the recognition site for cleavage of the cytosolic from membrane-associated portions of C is homologous for the YF NS2B-3 enzyme. At the C/pr-M junction, the portion of the signalase recognition site upstream of the cleavage site is that of the backbone, YF, and the portion downstream of the cleavage site is that of the insert, JE. At the E/NS1 junction, the portion of the signalase recognition site upstream of the cleavage site is similar to that of the insert, JE (four of five of the amino acids are identical to those of the JE sequence), and the portion downstream of the cleavage site is that of the backbone, YF. It is preferable to maintain this or a higher level of amino acid sequence identity to the viruses that form the chimera. Alternatively, at least 25, 50, or 75% sequence identity can be maintained in the three to five amino acid positions flanking the signalase and NS2B-3 protease recognition sites. Also possible is the use of any of numerous known signal sequences to link the C and pre-M or E and NS1 proteins of the chimeras (see, e.g., von Heijne, Eur. J. Biochem. 133:17-21, 1983; von Heijne, J. Mol. Biol. 184:99-105, 1985) or, for example, using the known sequences for guidance, one skilled in the art can design additional signal sequences that can be used in the chimeras of the invention. Typically, for example, the signal sequence will include as its last residue an amino acid with a small, uncharged side chain, such as alanine, glycine, serine, cysteine, threonine, or glutamine. Other requirements of signal sequences are known in the art (see, e.g., von Heijne, 1983, supra; von Heijne, 1985, supra). Construction of cDNA Templates for Generation of YF/JE Chimeric Virus

The derivation of full-length cDNA templates for YF/JE chimeras of the invention described below employed a strategy similar to that earlier workers used to regenerate YF 17D from cDNA for molecular genetic analysis of YF replication. The strategy is described, e.g., by Nestorowicz et al. (Virology 199:114-123, 1994).

Briefly, derivation of a YF/JE chimera of the invention involves the following. YF genomic sequences are propagated in two plasmids (YF5'3'IV and YFM5.2), which encode the YF sequences from nucleotides 1-2,276 and 8,279-10,861 (YF5'3'IV) and from 1,373-8,704 (YFM5.2) (Rice et al., The New Biologist 1:285-296, 1989). Full-length cDNA templates are generated by ligation of appropriate restriction fragments derived from these plasmids. This method has been the most reliable for ensuring stable expression of YF sequences and generation of RNA transcripts of high specific infectivity.

Our strategy for construction of chimeras involves replacement of YF sequences within the YF5'3'IV and YFM5.2 plasmids by the corresponding JE sequences from the start of the prM protein (nucleotide 478, amino acid 128) through the E/NS1 cleavage site (nucleotide 2,452, amino acid 817). In addition to cloning of JE cDNA, several steps were required to introduce or eliminate restriction sites in both the YF and JE sequences to permit in vitro ligation. The structure of the template for regenerating chimeric YF (C)/JE (prM-E) virus is shown in FIG. 2. A second chimera, encoding the entire JE structural region (C-prM-E) was engineered using a similar strategy.

#### Molecular Cloning of the JE Virus Structural Region

Clones of authentic JE structural protein genes were generated from the JE SA<sub>14</sub>-14-2 strain (JE live, attenuated vaccine strain), because the biological properties and molecular characterization of this strain are well-

documented (see, e.g., Eckels et al., Vaccine 6:513-518, 1988; JE SA<sub>14</sub>-14-2 virus is available from the Centers for Disease Control, Fort Collins, Colo. and the Yale Arbovirus Research Unit, Yale University, New Haven, Conn., which are World Health Organization-designated Reference Centers for Arboviruses in the United States). JE SA<sub>14</sub>-14-2 virus at passage level PDK-5 was obtained and passaged in LLC-MK<sub>2</sub> cells to obtain sufficient amounts of virus for cDNA cloning. The strategy used involved cloning the structural region in two pieces that overlap at an NheI site (JE nucleotide 1,125), which can then be used for in vitro ligation.

RNA was extracted from monolayers of infected LLC-MK<sub>2</sub> cells and first strand synthesis of negative sense cDNA was carried out using reverse transcriptase with a negative sense primer (JE nucleotide sequence 2,456-71) containing nested XbaI and NarI restriction sites for cloning initially into pBluescript II KS(+), and subsequently into YFM5.2 (NarI), respectively. First strand cDNA synthesis was followed by PCR amplification of the JE sequence from nucleotides 1,108-2,471 using the same negative sense primer and a positive sense primer (JE nucleotides sequence 1,108-1,130) containing nested XbaI and NsiI restriction sites for cloning into pBluescript and YFM5.2(NarI), respectively. JE sequences were verified by restriction enzyme digestion and nucleotide sequencing. The JE nucleotide sequence from nucleotides 1 to 1,130 was derived by PCR amplification of negative strand JE cDNA using a negative sense primer corresponding to JE nucleotides 1,116 to 1,130 and a positive sense primer corresponding to JE nucleotides 1 to 18, both containing an EcoRI restriction site. PCR fragments were cloned into pBluescript and JE sequences were verified by nucleotide sequencing. Together, this represents cloning of the JE sequence from nucleotides 1-2,471 (amino acids 1-792).

#### Construction of YF5'3'IV/JE and YFM5.2/JE Derivatives

To insert the C terminus of the JE envelope protein at the YF E/NS1 cleavage site, a unique NarI restriction site was introduced into the YFM5.2 plasmid by oligonucleotide-directed mutagenesis of the signalase sequence at the E/NS1 cleavage site (YF nucleotides 2,447-2,452, amino acids 816-817) to create YFM5.2(NarI). Transcripts derived from templates incorporating this change were checked for infectivity and yielded a specific infectivity similar to the parental templates (approximately 100 plaque-forming units/250 nanograms of transcript). The JE sequence from nucleotides 1,108 to 2,471 was subcloned from several independent PCR-derived clones of pBluescript/JE into YFM5.2(NarI) using the unique NsiI and NarI restriction sites. YF5'3'IV/JE clones containing the YF 5' untranslated region (nucleotides 1-118) adjacent to the JE prM-E region were derived by PCR amplification.

To derive sequences containing the junction of the YF capsid and JE prM, a negative sense chimeric primer spanning this region was used with a positive sense primer corresponding to YF5'3'IV nucleotides 6,625-6,639 to generate PCR fragments that were then used as negative sense PCR primers in conjunction with a positive sense primer complementary to the pBluescript vector sequence upstream of the EcoRI site, to amplify the JE sequence (encoded in reverse orientation in the pBluescript vector) from nucleotide 477 (N-terminus of the prM protein) through the NheI site at nucleotide 1,125. The resulting PCR fragments were inserted into the YF5'3'IV plasmid using the NotI and EcoRI restriction sites. This construct contains the SP6 promoter preceding the YF 5'-untranslated region, followed by the sequence: YF (C) JE (prM-E), and contains the NheI site (JE nucleotide 1,125) required for in vitro ligation.

#### Engineering YFM5.2 and YF5'3'IV to Contain Restriction Sites for In Vitro Ligation

To use the NheI site within the JE envelope sequence as a 5' in vitro ligation site, a redundant NheI site in the YFM5.2 plasmid (nucleotide 5,459) was eliminated. This was accomplished by silent mutation of the YF sequence at nucleotide 5,461 (T→C; alanine, amino acid 1820). This site was incorporated into YFM5.2 by ligation of appropriate restriction fragments and introduced into YFM5.2(NarI)/JE by exchange of an NsiI/NarI fragment encoding the chimeric YF/JE sequence.

To create a unique 3' restriction site for in vitro ligation, a BspEI site was engineered downstream of the AatII site normally used to generate full-length templates from YF5'3'IV and YFM5.2. (Multiple AatII sites are present in the JE structural sequence, precluding use of this site for in vitro ligation.) The BspEI site was created by silent mutation of YF nucleotide 8,581 (A→C; serine, amino acid 2,860), and was introduced into YFM5.2 by exchange of appropriate restriction fragments. The unique site was incorporated into YFM5.2/JE by exchange of the XbaI/SphI fragment, and into the YF5'3'IV/JE(prM-E) plasmids by three-piece ligation of appropriate restriction fragments from these parent plasmids and from a derivative of YFM5.2 (BspEI) deleting the YF sequence between the EcoRI sites at nucleotides 1 and 6,912.

#### Exchange of JE Nakayama cDNA into YF/JE Chimeric Plasmids

Because of uncertainty about the capacity of the PCR-derived JE SA<sub>14</sub>-14-2 structural region to function properly in the context of the chimeric virus, we used cDNA from a clone of the JE Nakayama strain that has been extensively characterized in expression experiments and for its capacity to induce protective immunity (see, e.g., McIdea et al., Virology 158:348-360, 1987; the JE Nakayama strain is available from the Centers for Disease Control, Fort Collins, Colo., and the Yale Arbovirus Research Unit, Yale University, New Haven, Conn.). The Nakayama cDNA was inserted into the YF/JE chimeric plasmids using available restriction sites (HindIII to PvuII and BpmI to MunI) to replace the entire prM-E region in the two plasmid system except for a single amino acid, serine, at position 49, which was left intact in order to utilize the NheI site for in vitro ligation. The entire JE region in the Nakayama clone was sequenced to verify that the replaced cDNA was authentic (Table 1).

#### Generation of Full-Length cDNA Templates, RNA Transfection, and Recovery of Infectious Virus

Procedures for generating full-length cDNA templates are essentially as described in Rice et al. (The New Biologist 1:285-96, 1989; also see FIG. 2). In the case of chimeric templates, the plasmids YF5'3'IV/JE(prM-E) and YFM5.2/JE are digested with NheI/BspEI and in vitro ligation is performed using 50 nanograms of purified fragments in the presence of T4 DNA ligase. The ligation products are linearized with XhoI to allow run-off transcription. SP6 transcripts are synthesized using 50 nanograms of purified template, quantitated by incorporation of <sup>3</sup>H-UTP, and integrity of the RNA is verified by non-denaturing agarose gel electrophoresis. Yields range from 5 to 10 micrograms of RNA per reaction using this procedure, most of which is present as full-length transcripts. Transfection of RNA transcripts in the presence of cationic liposomes is carried out as described by Rice et al. (supra) for YF 17D. In initial experiments, LLC-MK<sub>2</sub> cells were used for transfection and quantitation of virus, since we have determined the permissiveness for replication and plaque formation of the parental

strains of YF and JE. Table 2 illustrates typical results of transfection experiments using Lipofectin (GIBCO/BRL) as a transfection vehicle. Vero cell lines have also been used routinely for preparation of infectious virus stocks, characterization of labeled proteins, and neutralization tests.

Amplification products from Vero cells were sent to the FDA (CBER) for preparation of the RMS in diploid, fetal rhesus lung cells. Fetal rhesus lung cells were received from the ATCC as cultured cells and were infected with YF/JE SA<sub>14</sub>-14-2 (clone A-1) at an MOI of 1.0. After 1 hour of incubation at 37° C., the inoculum was aspirated and replaced with 50 ml of EMEM, containing 2% FBS. Virus was harvested 78 hours later, aliquoted into 1 ml vials (a total of 200 vials) and frozen at -70° C. Virus titers were determined in Vero, LLC MK2, and CV-1 cells using a standard plaque assay. Titers (pfu/ml) were 1.6×10<sup>6</sup> in Vero cells, 1.25×10<sup>6</sup> in LLC MK2 cells, and 1.35×10<sup>5</sup> in CV-1 cells.

#### Nucleotide Sequencing of Chimeric cDNA Templates

Plasmids containing the chimeric YF/JE cDNA were subjected to sequence analysis of the JE portion of the clones to identify the correct sequences of the SA<sub>14</sub>-14-2 and Nakayama envelope protein. The nucleotide sequence differences between these constructs in comparison to the reported sequences (McAda et al., supra) are shown in Table 1.

Five amino acid differences at positions 107, 138, 176, 264, and 279 separate the virulent from the attenuated strains of JE virus. Amino acid differences map to three subregions of Domains I and II of the flavivirus E protein model (Rey et al., *Nature* 375:291-298, 1995). These include the putative fusion peptide (position 107), the hinge cluster (positions 138, 279), the exposed surface of Domain I (positions 176 and 177), and the alpha-helix located in the dimerization Domain II (position 264). Changes at position 107, 138, 176, and 279 were selected early in the passage history, resulting in attenuation of JE SA<sub>14</sub>-14-2, and remained stable genetic differences from the SA<sub>14</sub>-14-2 parent (Ni et al., *J. Gen. Virol.* 75:1505-1510, 1994), showing that one or more of these mutations are critical for the attenuation phenotype. The changes at positions 177 and 264 occurred during subsequent passage, and appear to be genetically unstable between two SA<sub>14</sub>-14-2 virus passages in PHK and PDK cells, showing that this mutation is less critical for attenuation.

The nucleotide sequence of the E protein coding region of the RMS was determined to assess potential sequence variability resulting from viral passage. Total RNA was isolated from RMS-infected Vero cells, reversed transcribed, and PCR amplified to obtain sequencing templates. Several primers specific for SA<sub>14</sub>-14-2 virus were used in individual sequencing reactions and standard protocols for cycle sequencing were performed.

Sequence data revealed two single nucleotide mutations in the RMS E protein, when compared to the published SA<sub>14</sub>-14-2 JE strain sequence data. The first mutation is silent, and maps to amino acid position 4 (CTT to CTG); the second is at amino acid position 243 (AAA to GAA) and introduces a change from lysine to glutamic acid. Both mutations identified are present in the sequence of the JE wild type strains Nakayama, SA14 (parent of SA<sub>14</sub>-14-2), and JaOArS982 (Sumiyoshi et al., *J. Infect. Dis.* 171:1144-1151, 1995); thus, they are unlikely to contribute to virulence phenotype.

We conclude that in vitro passage in FRhL cells to obtain the RMS did not introduce unwanted mutations in the E protein. Sequence comparison to wild-type JE virus strains,

including the parental strain (SA14), demonstrated that differences between RMS and SA<sub>14</sub>-14-2 sequence may be due to errors in the original analysis of the SA<sub>14</sub>-14-2 sequence.

#### 5 Structural and Biological Characterization of Chimeric YF/JE Viruses

The genomic structure of chimeric YF/JE viruses recovered from transfection experiments was verified by RT/PCR-based analysis of viral RNA harvested from infected cell monolayers. These experiments were performed to eliminate the possibility that virus stocks were contaminated during transfection procedures. For these experiments, first-pass virus was used to initiate a cycle of infection, to eliminate any possible artifacts generated by the presence of residual transfected viral RNA. Total RNA extracts of cells infected with either the YF/JE (prM-E)-SA<sub>14</sub>-14-2 or YF/JE (prM-E)-Nakayama chimera were subjected to RT/PCR using YF and JE-specific primers that allowed recovery of the entire structural region as two PCR products of approximately 1 kilobase in size. These products were then analyzed by restriction enzyme digestion using the predicted sites within the JE SA<sub>14</sub>-14-2 and Nakayama sequences that allow differentiation of these viruses. Using this approach, the viral RNA was demonstrated to be chimeric and the recovered viruses were verified to have the appropriate restriction sites. The actual C-prM boundary was then verified to be intact at the sequence level by cycle sequence analysis across the chimeric YF/JE C-prM junction.

The presence of the JE envelope protein in the two chimeras was verified by both immunoprecipitation with JE-specific antisera and by plaque reduction neutralization testing using YF and JE-specific antisera. Immunoprecipitation of <sup>35</sup>S-labeled extracts of LLC-MK<sub>2</sub> cells infected with the chimeras using a monoclonal antibody to the JE envelope protein showed that the JE envelope protein could be recovered as a 55 kDa protein, while the same antisera failed to immunoprecipitate a protein from YF-infected cells. Both JE and YF hyperimmune sera demonstrated cross-reactivity for the two envelope proteins, but the size difference between the proteins (YF=53 kDa, unglycosylated; JE=55 kDa, glycosylated) could reproducibly be observed. Use of YF monoclonal antibodies was not satisfactory under the immunoprecipitation conditions, thus, the specificity was dependent on the JE monoclonal antibodies in this analysis.

Plaque reduction neutralization testing (PRNT) was performed on the chimeric viruses and the YF and JE SA<sub>14</sub>-14-2 viruses using YF and JE-specific hyperimmune ascitic fluid (ATCC) and YF-specific purified IgG (monoclonal antibody 2E10). Significant differences in the 50% plaque reduction titer of these antisera were observed for the chimeras when compared to the control viruses in these experiments (Table 3). The YF/JE SA<sub>14</sub>-14-2 chimeric vaccine candidate, as well as the Nakayama chimera and SA<sub>14</sub>-14-2 viruses, were neutralized only by JE ascitic fluid, whereas YF 17D was neutralized in a specific fashion by YF ascites and the monoclonal antibody (Table 3). Thus, epitopes required for neutralization are expressed in the infectious chimeric YF/JE viruses, and are specific for the JE virus.

#### Growth Properties in Cell Culture

The growth capacity of the chimeras has been examined quantitatively in cell lines of both primate and mosquito origin. FIG. 3 illustrates the cumulative growth curves of the chimeras on LLC-MK<sub>2</sub> cells after low multiplicity infection (0.5 plaque-forming units/cell). In this experiment, YF5.2iv (cloned derivative) and JE SA<sub>14</sub>-14-2 (uncloned) viruses

were used for comparison. Both chimeric viruses reached a maximal virus yield of approximately one log higher than either parental virus. In the case of the YF/JE SA<sub>14</sub>-14-2 chimera, the peak of virus production occurred 12 hours later than the YF/JE Nakayama chimera (50 hours vs. 38 hours). The YF/JE Nakayama chimera exhibited considerably more cytopathic effects than the YF/JE SA<sub>14</sub>-14-2 chimera on this cell line.

A similar experiment was carried out in C6136 cells after low multiplicity infection (0.5 plaque-forming units/cell). FIG. 3 also illustrates the growth kinetics of the viruses in this invertebrate cell line. Similar virus yields were obtained at all points used for virus harvest in this experiment, further substantiating the notion that chimeric viruses are not impaired in replication efficiency.

Additional experiments showing the growth properties of RMS are shown in FIG. 4. Briefly, Vero cells were grown in EMEM, 1% L-Glutamine, 1% non-essential amino acid, and 10% FBS buffered with sodium bicarbonate. LLC-MK2 cells were purchased from the ATCC (CLL-7.1, passage 12) and were grown in the same medium as Vero cells. Cells were inoculated with the RMS virus at an MOI of 0.1. Supernatant fluid was sampled at 24 hour intervals for 7 days and frozen at -70° C. for subsequent plaque assay. Plaque assays were performed in 6-well plates. The RMS reached more than 8 log<sub>10</sub> pfu/ml in 5 days. In LLC-MK2 cells, the RMS grew slower and peaked (6 log<sub>10</sub> pfu/ml) at about 6 days.

Comparison of Growth Kinetics of the RMS (YF/JE SA<sub>14</sub>-14-2) with YF 17D Vaccine in MRC-5 Cells

An experiment was performed to assess the ability of the vaccine candidate to propagate in a cell line acceptable for human vaccines. Commercial Yellow Fever 17D vaccine (YF-VAX® (Yellow Fever 17D vaccine)) was obtained from Connaught Laboratories, Swiftwater, PA. MRC-5 (diploid human embryonal lung cells) were purchased from ATCC (171-CCL, Batch#: F-14308, passage 18) and grown in EMEM, 2 mM L-Gln, Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 10% FBS.

To compare growth kinetics of RMS (sequence appendices 2 (SEQ ID NO: 12) and 3 (SEQ ID NO: 13); Research Master Seed, YF/JE SA<sub>14</sub>-14-2; nucleotide sequence of ORF: CL nucleotides 119-421; Pr-M: nucleotides 422-982; E: nucleotides 983-2482; and non-structural proteins: 2483-10381); (amino acid sequence of ORF; C: amino acids 1-101; Pr-M: amino acids 102-288; E: amino acids 289-788; and non-structural proteins: amino acids 789-3421); (nucleotide sequence of RMS; the coding sequence is from nucleotide 119 to nucleotide 10382)) with YF-VAX® (Yellow Fever 17D vaccine), cells were grown to 90% confluency and infected with RMS or YF-VAX® (Yellow Fever 17D vaccine) at an MOI of 0.1 pfu. Since MRC-5 cells generally grow slowly, these cells were kept for 10 days post inoculation. Samples were frozen daily for 7-10 days and infectivity determined by plaque assay in Vero cells. YF-VAX® (Yellow Fever 17D vaccine) and the YF/JE chimera grew to modest titers in MRC-5 cells (FIG. 5). The peak titer was -4.7 log<sub>10</sub> pfu for YF-VAX® (Yellow Fever 17D vaccine) achieved on the propagate in a cell line acceptable for human vaccines. Commercial Yellow Fever 17D vaccine (YF-VAX® (Yellow Fever 17D vaccine)) was obtained from Connaught Laboratories, Swiftwater, Pa. MRC-5 (diploid human embryonal lung cells) were purchased from ATCC (171-CCL, Batch#: F-14308, passage 18) and grown in EMEM, 2 mM L-Gln, Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 10% FBS.

To compare growth kinetics of RMS (sequence appendices 2 and 3; Research Master Seed, YF/JE SA<sub>14</sub>-14-2; nucleotide sequence of ORF; C: nucleotides 119-421; Pr-M second day and was slightly lower, 4.5 log<sub>10</sub> pfu, for the RMS after 6 days.

Growth Curve of YF/JE SA<sub>14</sub>-14-2 in FRhL Cells With and Without IFN-inhibitors

Fetal rhesus lung cells were obtained from the ATCC and propagated as described for MRC-5 cells. Growth kinetics of the RMS were determined with and without interferon inhibitors.

Double-stranded RNA appears to be the molecular species most likely to induce interferon (IFN) in many virus infected cells. Induction of interferon apparently plays a significant role in the cellular defense against viral infection. To escape cellular destruction, many viruses have developed strategies to down-regulate induction of interferon-dependent activities. Sindbis virus and vesicular stomatitis virus have been shown to be potent IFN inducers. Using chick embryo cells, mouse L cells, and different viral inducers of IFN, it was shown that 2-aminopurine (2AP) and indomethacin (IM) efficiently and reversibly inhibit IFN action (Sekellick et al., J. Interferon Res. 5:651, 1985; Marcus et al., J. Gen. Virol. 69:1637, 1988).

To test whether inhibition of IFN (if present) in FRhL cells will increase the virus yield, we added 2AP at a concentration of 10 mM or IM at a concentration of 10 mg/ml to the FRhL cells at the time of infection with 0.1 or 0.01 MOI of RMS. Samples were taken daily and frozen for determination of virus infectivity by plaque assay. As shown in FIG. 6A, virus titers peaked on day 4 in the presence or absence of inhibitors. When cells were infected at 0.01 MOI (FIG. 6A), virus titer reached 2.65x10<sup>7</sup> pfu/ml on day 4 in the absence of inhibitors. In cells infected in the presence of IM, virus titer was increased about 2-fold, to 5.95x10<sup>7</sup> pfu/ml on day 4. This increase was more dramatic (4-fold) when 2AP was used (9.7x10<sup>7</sup> pfu/ml). Addition of IM did not increase virus yield when cells were infected at a higher MOI (0.1). A titer of 5.42x10<sup>7</sup> was reached without inhibitor and 3.45x10<sup>7</sup> was achieved in the presence of IM. Addition of 2AP increased virus yields to 1.1x10<sup>8</sup> pfu/ml by day 4 and only 1 log<sub>10</sub> pfu was lost in the following 3 days (9.5x10<sup>6</sup> pfu/ml on day 7) (FIG. 6B). We conclude from this experiment that the YF/JE SA<sub>14</sub>-14-2 vaccine candidate replicates to titers of -7.5 log<sub>10</sub>/ml in an acceptable cell substrate. The addition of interferon inhibitors can result in a modest increase in yields, but is not a requirement for vaccine production.

Neurovirulence Testing in Normal Adult Mice

The virulence properties of the YF/JE SA<sub>14</sub>-14-2 chimera was analyzed in young adult mice by intracerebral inoculation. Groups of 10 mice (4 week old male and female ICR mice, 5 each per group) were inoculated with 10,000 plaque-forming units of the YF/JE SA<sub>14</sub>-14-2 chimera, YF 17D 5.2 iv, or the Chinese vaccine strain JE SA<sub>14</sub>-14-2 and observed daily for 3 weeks. The results of these experiments are illustrated in FIG. 7. Mice receiving the YF5.2 iv parent succumbed by approximately one week post-inoculation. No mortality or illness was observed among mice receiving either the JE SA<sub>14</sub>-14-2 parent or the chimera. The inocula used for the experiments were titrated at the time of injection and a subgroup of the surviving mice were tested for the presence of neutralizing antibodies to confirm that infection had taken place. Among those tested, titers against the JE SA<sub>14</sub>-14-2 virus were similar for animals receiving either this strain or the chimera.

The results of additional experiments investigating the neurovirulence of the YF/JE SA<sub>14</sub>-14-2 chimera in mice are

illustrated in Table 4. In these experiments, all of the mice inoculated with YF5.2iv died within 7-8 days. In contrast, none of the mice inoculated with YF/JE SA<sub>14</sub>-14-2 died during two weeks of post-inoculation observation.

The results of experiments investigating the neuroinvasiveness and pathogenesis of YF/JE chimeras are illustrated in Table 5. In these experiments, the chimeric viruses were inoculated into 3 week old mice at doses varying between 10,000 and 1 million plaque-forming units via the intraperitoneal route. None of the mice inoculated with YF/JE Nakayama or YF/JE SA<sub>14</sub>-14-2 died during three weeks of post-inoculation observation, indicating that the virus was incapable of causing illness after peripheral inoculation. Mice inoculated with YF/JE SA<sub>14</sub>-14-2 developed neutralizing antibodies against JE virus (FIG. 8).

In additional experiments testing the neurovirulence phenotype and immunogenicity of the RMS, 4-week old ICR mice (n=5) were inoculated by the i.c. route with 0.03 ml of graded doses of the RMS or YF-VAX® (Yellow Fever 17D vaccine) (Table 6). Control mice received only diluent medium by this route. Mice were observed daily and mortality rates were calculated.

Mice inoculated with YF-VAX® (Yellow Fever 17D vaccine) started to die on day 7 (FIG. 9A). The icLD<sub>50</sub> of unpassaged YF-VAX® (Yellow Fever 17D vaccine), calculated by the method of Reed and Muench, was 1.62 log<sub>10</sub> and the average survival time (AST) at the highest dose (4.2 log<sub>2</sub> pfu) was 8.8 days. In contrast, all mice receiving the RMS survived challenge at all doses (FIG. 9B), indicating that the virus is not neurovirulent for mice. None of the mice inoculated with YF-VAX® (Yellow Fever 17D vaccine) or the RMS by the peripheral (subcutaneous) route (as shown in Table 6) showed signs of illness or death. Thus, as expected, yellow fever 17D virus was not neuroinvasive. Comparison of Immunogenicity of YF/JE RMS with YF 17D Vaccine

The immunogenicity of the of the RMS was compared with that of the YF 17D vaccine in outbred ICR mice. Groups of five 4 week-old mice received graded doses of the vaccines shown in Table 6. Mice were inoculated with 100 µl of each virus dilution by the s.c. route. For comparison, two groups of mice received two weekly doses of commercial inactivated JE vaccine prepared in mouse brain tissue (JE-VAX® (inactivated Japanese Encephalitis virus vaccine)) at 1:30 and 1:300 dilution, representing 10x and 1x the human equivalent dose based on body weight, respectively. Animals were bled 3 and 8 weeks later and neutralizing antibody titers were measured in heat-inactivated sera against homologous viruses by PRNT. End-point titers were the highest dilution of sera which reduced the number of viral plaques by 50% compared to a normal mouse serum control.

The highest N antibody titers were observed 8 weeks after immunization in mice receiving 5 log<sub>10</sub> pfu of the RMS (FIG. 10 and Table 7). The geometric mean N antibody titer in these mice was 5,614. N antibody responses induced by YF/JE SA<sub>14</sub>-14-2 vaccine against JE were higher than N antibody responses against YF induced by YF 17D vaccine. Interestingly, the highest concentration of the YF 17D vaccine did not induce significant titers of neutralizing antibodies 3 or 8 weeks post immunization, but antibodies were elicited at lower doses.

Very low doses (1.4-2.4 log<sub>10</sub> PFU) of YF 17D vaccine elicited an immune response in mice 8 weeks after inoculation (Table 7). This result may indicate delayed replication of the vaccine in mice receiving low virus inocula. In contrast, the YF/JE SA<sub>14</sub>-14-2 chimeric vaccine in this dose

range was not immunogenic. It is likely that the chimeric vaccine is somewhat less infectious for mice than YF 17D. However, when inoculated at an infective dose, the chimera appears to elicits a higher immune response. This may be due to higher replication in, or altered tropism for, host tissues. Animals that received two doses of JE-VAX® (inactivated Japanese Encephalitis virus vaccine) did not mount a significant antibody response. Only one animal in the 1:30 dose group developed a neutralizing titer of 1:10 eight weeks after immunization. This might be due to the route (s.c.) and dilution (1:30) of the vaccine.

#### Protection of YF/JE SA<sub>14</sub>-14-2 RMS Immunized Mice Against Challenge With Virulent JE

The YF/JE SA<sub>14</sub>-14-2 RMS and other viruses were evaluated for immunogenicity and protection in C57/BL6 mice in collaboration with Dr. Alan Barrett, Department of Pathology, University of Texas Medical Branch, Galveston. Experimental groups are shown in Table 8. Ten-fold dilutions (10<sup>2</sup>-10<sup>5</sup>) of each virus were inoculated by the s.c. route into groups of 8 mice. Mice were observed for 21 days, at which time surviving animals were bled from the retro-orbital sinus and serum frozen for neutralization tests. The 50% immunizing dose (ID<sub>50</sub>) for each virus and GMT was determined (see below).

Surviving mice that received viruses by the s.c. route were challenged on day 28 by i.p. inoculation of 158 LD<sub>50</sub> (2,000 PFU) of JE virus (JaOArS982, IC37). Animals were observed for 21 days following challenge. Protection is expressed as the proportion of mice surviving challenge (Table 9).

As expected, YF 17D virus afforded minimal cross-protection against JE challenge. The YF/JE SA<sub>14</sub>-14-2 RMS chimera was protective at doses ≥10<sup>3</sup> PFU. The 50% protective dose of the chimeric vaccine was 2.32 log<sub>10</sub> PFU. Animals that received 3 doses of JE-VAX® (inactivated Japanese Encephalitis virus vaccine) were solidly protected against challenge. Mice given a single dose of the SA<sub>14</sub>-14-2 vaccine were poorly protected. Wild-type Nakayama virus was lethal for a proportion of animals, in a dose-dependent fashion; survivors were poorly protected against challenge indicating that the lethal dose was close to the infecting dose for this virus.

The YF/JE<sup>Nakayama</sup> chimeric virus was somewhat more virulent than the Nakayama strain, in that all mice given 10<sup>5</sup> of the chimera died after inoculation. This is in contrast to earlier studies in outbred mice, in which this virus was not neuroinvasive, confirming the increased susceptibility of C57/BL6 mice to peripheral challenge with JE viruses. Survivors were fully protected against challenge, showing that the infection established by the chimeric virus was more active (immunogenic) than infection by Nakayama virus without the YF replication background. These results show that the combination of viral envelope determinants of a neurovirulent strain (Nakayama) with a replication-efficient virus (YF 17D) can enhance virulence of the recombinant, emphasizing the need for genetic stability of the mutations conferring attenuation in the YF/JE<sup>Nakayama</sup> chimera.

#### Serological Response

Sera from mice in groups shown in Table 8 were tested 21 days after immunization for neutralizing antibodies. N tests were performed as follows. Six-well plates were seeded with Vero cells at a density of 10<sup>6</sup> cells/well in MEM alpha containing 10% FBS, 1% nonessential amino acids, buffered with sodium bicarbonate. One hundred µl of each test serum (inactivated at 60° C. for 30 minutes) diluted two-fold was mixed with an equal volume of virus containing 200-300 PFU. The virus-serum mixtures were incubated at 4° C.



overnight and 100  $\mu$ l added to each well after removal of growth medium. The plates were overlaid after 1 hour incubation at 37° C. with 0.6% agarose containing 3% fetal calf serum, 1% L-glutamine, 1% HEPES, and 1% pen-strep-amphotericin mixed 1:1 with 2xM199. After 4 days of incubation at 37° C., 5% CO<sub>2</sub>, a second overlay containing 3% Neutral red was added. After appearance of plaques, the monolayer was fixed with 1% formaldehyde and stained with crystal violet. The plaque reduction titer  $\mu$ s determined as the highest dilution of serum inhibiting  $\geq$ 50% of plaques compared with the diluent-virus control.

Results are shown in Table 10 and FIG. 11. NT antibody responses in mice immunized with the YF/JE SA<sub>14</sub>-14-2 chimera showed a dose response and good correlation with protection. At doses of 4-5 logs, the chimeric vaccine elicited higher N antibody responses against JE than either SA<sub>14</sub>-14-2 virus or wild-type Nakayama virus. Responses were superior to those elicited by YF-VAX® (Yellow Fever 17D vaccine) against YF 17D virus. No prozone effect was observed in animals receiving the chimera or infectious-clone derived YF 5.2iv; responses at the highest vaccine dose (5 logs) were higher than at the next lower dose (4 logs). In contrast, mice that received SA<sub>14</sub>-14-2, Nakayama, and YF-VAX® (Yellow Fever 17D vaccine) at the highest dose responded less well than animals inoculated with diluted virus.

#### Safety and Immunogenicity of CHIMERIVAX™-JE (Chimeric Flavivirus Vaccine Comprising Japanese Encephalitis Virus prM and E Proteins) in Monkeys

The safety of RMS was tested in monkeys, essentially as described in WHO Biological Standards for YF 17D vaccine with minor modifications (see below). Two groups (N=3) of rhesus monkeys were bled and shown to be free from HI antibodies to YF, JE, and SLE. Group 1 received undiluted CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) (LLC MK2-PI, Vero-1 passage after transfection) by the I.C. route (frontal lobe). Group 2 (N=3) received 0.25 ml of 1:10 diluted commercial YF 17D vaccine (YF-VAX® (Yellow Fever 17D vaccine)) by the same route. The virus inocula were frozen, back titrated, and shown to contain 7.0 and 5.0 log<sub>10</sub> pfu/0.25 ml of YF/JE SA<sub>14</sub>-14-2 and YF-VAX® (Yellow Fever 17D vaccine), respectively.

Monkeys were observed daily for clinical signs and scored as in WHO standards. Sera were collected daily for 7 days after inoculations and tested for viremia by plaque assay in Vero cells. Blood collected 2 and 4 weeks post inoculation and tested for NT antibodies to the homologous viruses. None of the monkeys showed sign of illness. Monkeys were euthanized on Day 30, and brains and spinal cords were examined for neuropathology as described in the WHO standards. A sample of the brain and spinal cord from each animal was collected and stored frozen for virus isolation attempts and immunocytochemistry experiments.

As shown in FIG. 12, a low level viremia was detected in all animals in both groups, and lasted for 2-3 days for the RMS and 1-2 days for YF-VAX® (Yellow Fever 17D vaccine). All viruses were cleared from the blood by Day 4. According to the WHO standards, monkeys receiving 5,000-50,000 (3.7-4.7 log<sub>10</sub>) pfu should not have viremia greater than 165,000 pfu/ml (approximately 16,500 mL<sub>50</sub>). None of the monkeys in the experiments had viremia of more than 15,000 pfu/ml, despite receiving 6 log<sub>10</sub> pfu of the RMS.

Neutralizing antibody titers were measured at 2 and 4 weeks post inoculation (FIG. 13). All monkeys seroconverted and had high titers of neutralizing antibodies against

the inoculated viruses. The level of neutralizing antibodies in 2 of 3 monkeys in both groups exceeded a titer of 1:6,400 (the last dilution of sera tested) at 4 weeks post inoculation. The geometric mean antibody titers for CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) were 75 and 3,200 after 2 and 4 weeks respectively and were 66 and 4971 for the YF-VAX® (Yellow Fever 17D vaccine) for the same time points (Table 11).

Histopathological examination of coded specimens of brain and spinal cord were performed by an expert neuropathologist (Dr. I. Levenbook, previously CBER/FDA), according to the WHO biological standards for yellow fever vaccine. There were no unusual target areas for histopathological lesions in brains of monkeys inoculated with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins). Mean lesion scores in discriminator areas were similar in monkeys inoculated with YF-VAX® (Yellow Fever 17D vaccine) (0.08) and monkeys inoculated with a 100-fold higher dose of CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) (0.07). Mean lesion scores in discriminator+target areas were higher in monkeys inoculated with YF-VAX® (Yellow Fever 17D vaccine) (0.39) than in monkeys inoculated with a 100-fold higher dose of CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) (0.11). These preliminary results show an acceptable neurovirulence profile and immunogenicity for CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) vaccine. A summary of the histopathology results is provided in Table 22.

#### Efficacy of YF/JE Chimera in Protecting Monkeys Against Intracerebral Challenge

The YF/JE chimera were given to adult rhesus monkeys without pre-existing flavivirus immunity by the subcutaneous route. Three monkeys received 4.3 log pfu and three monkeys received 5.3 log pfu of YF/JE SA<sub>14</sub>-14-2 virus. All 6 monkeys developed very low level (1-2 log/ml) viremias. All animals developed neutralizing antibodies by day 15 (earliest time tested) and titers rose by day 30. Five of six animals survived a very severe intracerebral challenge with a highly virulent JE virus (100,000 mouse LD<sub>50</sub> were injected IC 60 days after immunization). None of 4 sham immunized monkeys survived; all died between days 8-10 after challenge. The single death in the immunized group was a pregnant female; pregnancy could have suppressed the cellular immune response to the vaccine. The results show the immunogenicity and protective efficacy of the vaccine, while validating safety with respect to low vaccine viremia. The results of these experiments are illustrated in Tables 12-15.

#### Genetic Stability of the RMS

The E protein of the attenuated SA<sub>14</sub>-14-2 virus used to construct the YF/JE chimera differs from its virulent parent (SA 14 or Nakayama) at 6 positions; 107, 138, 176, 177, 264, and 279. Because the presence of a single residue controlling virulence would be a disadvantage for any vaccine candidate because of the potential for reversion, studies are being undertaken to determine which residue(s) are responsible for attenuation and in particular whether a single residue is responsible for the difference.

#### Position 138 on the E Protein

A single mutation of an acidic residue glutamic acid (E) to a basic residue, lysine (K) at position 138 on the E protein of JE virus results in attenuation (Sumiyoshi et al., J. Infect.



Dis. 171:1144, 1995). Experiments were carried out to determine whether the amino acid at position 138 of the JE envelope protein (K in the vaccine chimera and E in the virulent Nakayama chimera) is a critical determinant for neurovirulence in mice. Chimeric YF/JE SA<sub>14</sub>-14-2 (K 138---->E) virus containing the single reversion of K---->E at position 138 was generated from an engineered cDNA template. The presence of the substitution and the integrity of the entire E protein of the resulting virus was verified by RT/PCR sequencing of the recovered virus. A standard fixed-dose neurovirulence test of the virus was conducted in 4-week-old outbred mice by i.c. inoculation with 10<sup>6</sup> pfu of virus. The YF/JE SA<sub>14</sub>-14-2 and YF/JE Nakayama chimeric viruses were used as controls. The virulence phenotype of YF/JE SA<sub>14</sub>-14-2 (K---->E) was indistinguishable from that of its attenuated parent YF/JE SA<sub>14</sub>-14-2 in this assay, with no morbidity or mortality observed in the mice during the observation period (FIG. 14).

We conclude that the single mutation at position 138 to the residue found in the JE-Nakayama virus does not exert a dominant effect on the neurovirulence of the YF/JE SA<sub>14</sub>-14-2 chimera, and that one or more additional mutations are required to establish the virulent phenotype.

#### Other Putative Attenuation Loci

Additional experiments to address the contributions of the other 6 residues (mentioned above) using the format described here were conducted. The mutant viruses constructed by site directed mutagenesis of the YF and JE infectious clones are listed in Table 16. The E proteins of these viruses were sequenced and confirmed to contain the desired mutations. Upon inoculation into weanling mice by the I.C. route it is possible to determine those residues involved in attenuation of the vaccine.

Additional experiments to address the contributions of other residues are underway. The mutant viruses constructed to date by site-directed mutagenesis of the YF and JE infectious clones are listed in Table 16. The methodology is as described above. Results to date confirm that at least two and possibly more than 2 mutations are responsible for the attenuation phenotype of YF/JE SA<sub>14</sub>-14-2 virus (Table 23). Stability of the RMS in Tissue Cultures: Characterization of Genetic Changes, Neurovirulence and Immunogenicity Serial Passages In Vitro

The RMS was used to inoculate a T75 flask of FRhL2 cells at an m.o.i. of 0.1. Subsequent passages were carried out in T75 flasks and harvested 3 days post-inoculation. At each passage, the culture supernatant was assumed to hold 10<sup>7</sup> pfu/ml and an aliquot corresponding to an moi of approximately 0.1 was added to a fresh flask of cells. The remainder of the culture supernatant was stored at -80° C. for later characterization.

#### Quasispecies and DNA Sequencing

The chimeric JE vaccine is an RNA virus. Selective pressure can cause rapid changes in the nucleic acid sequences of RNA viruses. A mutant virus that invades FRhL cells more rapidly, for example, may gain a selective advantage by competing more effectively with the original vaccine virus and take over the culture. Therefore, mutant strains of the vaccine that grow better than the original vaccine may be selected by subculturing in vitro. One concern that addressed experimentally is whether such selective pressures might lead to mutant vaccine viruses with increased virulence.

In theory, molecular evolution should occur more rapidly for RNA viruses than DNA viruses because viral RNA polymerases have higher error rates than viral DNA poly-

merases. According to some measurements, RNA virus mutation rates approach one mutation per. replication event. This is why an RNA virus can be thought of as a family of very closely related sequences (or "quasispecies"), instead of a single unchanging sequence (a "classical species").

Two different approaches can be taken to determine the sequence of an RNA virus:

- 1) purify viral genomic RNA from the culture supernatant, reverse-transcribe the RNA into cDNA and sequence this cDNA. This is the approach we have taken. It yields an averaged, or consensus sequence, such that only mutations which represent a large proportion (roughly, >20%) of the viruses in the culture can be detected.
- 2) Alternatively, cDNA can be cloned and individual clones sequenced. This approach would reveal the quasispecies nature of the vaccine by identifying individual mutations (deviations from the consensus sequence) in some proportion of the clones.

#### Biological Characterization of Serially Passaged RMS

As stated above, we demonstrated experimentally that the selective pressures exerted by serial passaging of the RMS does not lead to mutant vaccine viruses with increased virulence. Here, three biological properties of Passages 10 and 18 (P10 and P18) were examined. First, neurovirulence was tested by inoculating mice i.c. with graded doses of P1 as well as P10 and P 18. Second, immunogenicity was compared by inoculating mice s.c. with graded doses of the RMS, P10 and P18. Blood was drawn from these mice 30 days post inoculation and serum neutralizing titers were determined and compared. Finally, the growth kinetics of the RMS and of P10 and P18 were compared by inoculating FRhL cells at moi's of 0.1 and 0.01 and collecting samples of culture supernatant daily. The titers in each flask were plotted as a function of time and compared.

#### Stability of prM and E Genes

The M and E genes of P1 0 and P18 were sequenced completely from base 642 to base 2454. Both sequences were identical and carried only one mutation (A->G) resulting an amino acid substitution from H to R at position 394 on the E protein. This means that selective pressures did not lead to the loss of any of the attenuating mutations of the E gene. Codon H394 (CAC) encodes a Histidine in the RMS but we have found that the second base of this codon is mutated to a G in a significant proportion of the viruses, leading to the expression of Arginine. It is important to emphasize that a mixture of A and G are observed at this position in the sequence data. The ratio of A to G (A/G) was also determined for P1, P4, and P8. Interestingly, the ratio decreases steadily from P1 to P10, but at P18 it is back to the value seen at P8. One possible explanation for this observation is that a mutant bearing the H394R mutation gradually became as abundant as the original virus but was then out-competed by a new mutant bearing other mutations not present in the M or E genes and therefore, only detected as a rebound in the A/G ratio. We are reproducing these results by doing a second passaging experiment under identical conditions. It must also be noted that duplicate samples of viral genomic RNA were isolated, reverse-transcribed, amplified, and sequenced in parallel for each passage examined. Reported results were seen in both duplicate samples, arguing against any RT-PCR artifacts obscuring the data.

These observations show that minor genetic changes (one nucleotide substitution in the entire envelope E and M genes) have occurred in the YF sequences of the chimeric vaccine upon passaging, but that selective pressures did not lead to the loss of any of the attenuating mutations of the E gene.

## Neurovirulence Phenotype of Passages 10 and 18

Groups of five female ICR mice, 3 to 4 weeks-old, received 30  $\mu$ l i.c. of undiluted, P1, P10, or P18, as well as 30  $\mu$ l of 10-fold dilutions. None of the mice injected with P1, P10, or P18 (doses  $\geq 7 \log_{10}$  pfu) showed any sign of illness over a five week period. As determined by back-titration, the doses administered (pfu) were measured as shown in Table 17.

## Immunogenicity of Passages 10 and 18

Groups of five female ICR mice were injected subcutaneously (s.c.) with 100  $\mu$ l of undiluted virus stock of either the RMS or P10 or P18, as well as with doses of  $10^5$  and  $10^4$  pfu (see Table 18, results of back-titration).

## Growth Kinetics of Passages 10 and 18

Monolayers (90% confluent) of FRhL cells were infected with an moi of 0.1 or 0.01 of RMS, P10, or P18. Time points were then taken daily for seven days and the titer of each time point was determined by plaque assay. Visual observation of cytopathic effects (CPE) on FRhL cells used in this growth curve experiment show that later passages of the RMS have different growth properties than the RMS itself. CPE is clearly greater for P18 and P10 than for the RMS at 4 days postinfection showing that these viruses might replicate much faster than the RMS.

Other observations also show that the growth properties of P10 and P18 differ from those of the RMS. The titers of P1, P10, and P18 are  $\sim 2 \times 10^7$ ,  $2 \times 10^8$ , and  $3 \times 10^8$ , respectively. The relative yields of RT-PCR products suggest higher titers of P10 and P18 compared to P1. Although the PCR data are not necessarily quantitative, they are consistent with the observed titers.

These results raise the possibility that we have discovered a completely attenuated and probably immunogenic variant of the vaccine that grows to titers ten-fold higher than the original vaccine (RMS) in tissue culture. Such a mutant may have value for manufacturing.

Finally, the sequences of the entire genomes of the RMS and p18 were determined and found to be identical, except for the E-H394 mutation (Table 25). There are 6 nucleotide (NT) differences (NT positions are shaded) between the published YF 17D sequences and RMS shown in bold letters. Changes in positions 5461, 5641, 8212, and 8581 are silent and do not result in amino acid substitution, whereas changes in positions 4025 (ns2a) and 7319 (ns4b) result in amino acid substitutions from V to M and from E to K, respectively. Amino acid Methionine (M) at position 4025 is unique for RMS and is not found in any other YF strains, including parent Asibi virus and other yellow fever 17D strains (e.g., 204, 213, and 17DD), whereas Lysine (K) at position 7319 is found in 17D204F, 17D213, and 17DD, but not in 17D204US or Asibi strain. Since the RMS is more attenuated than YF 17D with respect to neurovirulence, and thus has better biological attributes as a human vaccine, it is possible that the amino acid differences at positions 4025 and 7319 in the nonstructural genes of the yellow fever portion of the chimeric virus contribute to attenuation. Other workers have shown that the nonstructural genes of yellow fever virus play an important role in the attenuation of neurovirulence (Monath, "Yellow Fever," in Plotkin et al., (Eds.), Vaccines, 2<sup>nd</sup> edition, W. B. Saunders, Philadelphia, 1998).

Experiment to Identify Possible Interference Between YF 17D and YF/JE SA<sub>14</sub>-14-2

It is well-established that yellow fever virus encodes antigenic determinants on the NS1 protein that induce non-neutralizing, complement-fixing antibodies. Passive immunization of mice with monoclonal anti-NS 1 antibodies

confers protection against challenge. Active immunization with purified or recombinant NS1 protects mice and monkeys against lethal challenge. The mechanism of protection is presumed to involve antibody-mediated complement-dependent cytotoxicity.

In addition to protective determinants on NS 1, CTL epitopes on other nonstructural proteins, including NS3, NS2a, and possibly NS5 may be involved in protection. Thus, infection with the YF/JE chimeric virus may stimulate humoral or cellular anti-yellow fever immunity. It is possible, therefore, that use of the chimeric vaccine may interfere with subsequent immunization against YF 17D, or that prior immunization with YF 17D may interfere with seroconversion to YF/JE SA<sub>14</sub>-14-2. Against this hypothesis is a substantial body of data showing that reimmunization with YF 17D results in a boost in yellow fever N antibodies. Those data show that it should be possible to successfully immunize against JE in an individual with prior YF immunity and vice versa.

To investigate possible interference effects, the experiment shown in Table 19 was initiated. Mice are immunized with one vaccine and subsequently boosted with the heterologous vaccine. Mice are bled every 30 days and sera tested for neutralizing antibodies against heterologous and homologous viruses.

## Seroconversion Rate and Antibody Titers After Primary Immunization

Three groups (n=8) of 34 weeks old female outbred ICR mice were immunized with a single dose (5.3  $\log_{10}$  pfu) of CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) (YF/JE SA<sub>14</sub>-14-2), three groups (n=8) were immunized with two doses of JE-VAX® (inactivated Japanese Encephalitis virus vaccine) (0.5 ml of a 1:5 dilution of reconstituted vaccine) and three groups (n=8) were immunized with a single dose of YF-VAX® (Yellow Fever 17D vaccine) (0.1 ml of a 1:2 dilution of reconstituted vaccine, containing 4.4  $\log_{10}$  pfu, previously determined to induce the highest immune response to YF virus). Six groups (n=4) of mice (similar age, 3-4 weeks old) were kept as controls for booster doses at 3, 6, and 12 months post primary immunization.

All mice were bled 4 and 8 weeks after primary immunization and their neutralizing antibody titers were measured against homologous viruses in a plaque assay. 21/24 (87.5%) of the animals immunized with a single dose of CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) developed anti-JE neutralizing antibodies 1 month after immunization; at 2 months, 18/24 (75%) were seropositive. Geometric mean increased somewhat between 1 and 2 months post inoculation. In contrast, only 25%-33% of the mice immunized with YF-VAX® (Yellow Fever 17D vaccine) seroconverted and antibody responses were low. These results show that YF 17D virus and chimeric viruses derived from YF 17D are restricted in their ability to replicate in the murine host; however, when the envelope of JE virus is incorporated in the chimeric virus, the ability to replicate in and immunize mice is apparently enhanced. Mice receiving two doses of JE-VAX® (inactivated Japanese Encephalitis virus vaccine) developed high neutralizing titers against parent Nakayama virus, and titers increased between 1 and 2 months post immunization.

Secondary Immunization of CHIMERIVAX™-JE (Chimeric Flavivirus Vaccine Comprising Japanese Encephalitis Virus prM and E Proteins) and JE-VAX® (Inactivated Japanese Encephalitis Virus Vaccine)-Immunized Mice With YF—VAX® (Yellow Fever 17D vaccine)

Three months and six months after primary immunization with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins), mice were inoculated with YF-VAX® (Yellow Fever 17D Vaccine) (1:2 dilution of a human dose containing  $4.4 \log_{10}$  pfu). Control mice not previously immunized and of identical age received CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) only or YF-VAX® (Yellow Fever 17D Vaccine) (Groups 10-13). One month later, mice were tested for presence of YF-specific neutralizing antibodies.

At the 3 month time point, none of the control mice or mice previously immunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) or JE-VAX® (inactivated Japanese Encephalitis virus vaccine) seroconverted to YF-VAX® (Yellow Fever 17D Vaccine), again confirming the poor immunogenicity of YF-VAX® (Yellow Fever 17D Vaccine) at the dose used. However, all mice immunized with YF-VAX® (Yellow Fever 17D Vaccine) 6 months after primary immunization with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) and 7/8 mice previously immunized with JE-VAX® (inactivated Japanese Encephalitis virus vaccine), seroconverted after immunization with YF-VAX® (Yellow Fever 17D Vaccine) (Table 24). There was no difference in seroconversion rate or GMT in mice with and without prior immunization with either JE vaccine. Secondary Immunization of YF-VAX® (Yellow Fever 17D Vaccine) Immunized Mice with CHIMERIVAX™-JE (Chimeric Flavivirus Vaccine Comprising Japanese Encephalitis Virus prM and E Proteins)

All mice previously immunized with YF-VAX® (Yellow Fever 17D Vaccine) and reimmunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) 3 months later developed neutralizing antibodies to JE (group 7, Table 10.4). None of the controls seroconverted. Five of 6 mice (83%) previously immunized to YF-VAX® (Yellow Fever 17D Vaccine) and reimmunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) 6 months later seroconverted to JE (group 8, Table 10.4, as did all controls (group 13)), and the GMTs were similar across these groups.

There was no evidence for cross-protection between YF and JE viruses or limitation of antibody response to sequential vaccination with these viruses. Yellow fever 17D vaccine elicits a poor antibody response in the mouse; while this limited interpretation of the data somewhat, it provided a sensitive test of any restriction in replication and immunogenicity of YF 17D virus in mice previously immunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins). The fact that all mice immunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) responded 6 months later to immunization with YF-VAX™ (Yellow Fever 17D Vaccine) and that the GMT and range of neutralizing antibody titers were similar to controls suggests that the chimeric vaccine imposed no significant barrier to yellow fever immunization. Construction of cDNA Templates for Generation of Yellow Fever/Dengue (YF/DEN) Chimeric Viruses

Derivation of chimeric Yellow Fever/Dengue (YF/DEN) viruses is described as follows which, in principle, is carried out the same as construction of the YF/JE chimeras described above. Other flavivirus chimeras can be engi-

neered with a similar strategy, using natural or engineered restriction sites and, for example, oligonucleotide primers as shown in Table 20.

#### Construction of YF/DEN Chimeric Virus

Although several molecular clones for dengue viruses have been developed, problems have commonly been encountered with stability of viral cDNA in plasmid systems, and with the efficiency of replication of the recovered virus. We chose to use a clone of DEN-2 developed by Dr. Peter Wright, Dept. of Microbiology, Monash University, Clayton, Australia, because this system is relatively efficient for regenerating virus and employs a two-plasmid system similar to our own methodology. (See Table 21 for a comparison of the sequences of Dengue-2 and YF/DEN-2<sub>218</sub> viruses.) The complete sequence of this DEN-2 clone is available and facilitated the construction of chimeric YF/DEN templates because only a few modifications of the YF clone were required. The relevant steps are outlined as follows.

Similar to the two plasmid system for YF5.2iv and YF/JE viruses, the YF/DEN system uses a unique restriction site within the DEN-2 envelope protein (E) as a breakpoint for propagating the structural region (prM-E) within the two plasmids, hereinafter referred to as YF5'3'IV/DEN (prM-E') and YFM5.2/DEN (E'-E) (see FIG. 15). The two restriction sites for in vitro ligation of the chimeric template are AatII and SphI. The recipient plasmid for the 3' portion of the DEN E protein sequence is YFM5.2(NarI[+]SphI[-]). This plasmid contains the NarI site at the E/NSI junction, which was used for insertion of the carboxyl terminus of the JE E protein. It was further modified by elimination of an extra SphI site in the NS5 protein region by silent site-directed mutagenesis. This allowed insertion of DEN-2 sequence from the unique SphI site to the NarI site by simple directional cloning. The appropriate fragment of DEN-2 cDNA was derived by PCR from the DEN-2 clone MON310 furnished by Dr. Wright. PCR primers included a 5' primer flanking the SphI site and a 3' primer homologous to the DEN-2 nucleotides immediately upstream of the signalase site at the E/NSI junction and replacing the signalase site by substitutions that create a novel site, but also introduce a NarI site. The resulting 1,170 basepair PCR fragment was then introduced into YFM5.2(NarI[+]SphI[-]).

The 5' portion of the DEN-2 clone including the prM and amino terminal portion of the E protein was engineered into the YF5'3'IV plasmid using a chimeric PCR primer. The chimeric primer, incorporating the 3' end of negative-sense YF C protein and 5' end of DEN-2 prM protein, was used with a positive-sense primer flanking the SP6 promoter of the YF5'3'IV plasmid to generate a 771 basepair PCR product with a 20 base extension representing DEN-2 prM sequence. This PCR product was then used to prime the DEN-2 plasmid in conjunction with a 3' primer representing DEN-2 sequence 1,501-1,522 and flanking the SphI, to generate an 1,800 basepair final PCR product including the YF sequence from the NotI site through the SP6 promoter, YF 5' untranslated region, and YF C protein, contiguous with the DEN-2-prM-E 1522 sequence. The PCR product was ligated into YF5'3'IV using NotI and SphI sites to yield the YF5'3'IV/DEN(prM-E) plasmid.

#### Construction of Chimeric YF/DEN Viruses Containing Portions of Two DEN Envelope Proteins

Since neutralization epitopes against DEN viruses are present on all three domains of the E protein, it is possible to construct novel chimeric virus vaccines that include sequences from two or more different DEN serotypes. In this embodiment of the invention, the C/prM junction and gene

encoding the carboxyl terminal domain (Domain III) of one DEN serotype (e.g., DEN-2) and the N-terminal sequences encoding Domains I and II of another DEN serotype (e.g., DEN-1) are inserted in the YF 17D cDNA backbone. The junctions at C/prM and E/NS1 proteins are retained, as previously specified, to ensure the infectivity of the double-chimera. The resulting infectious virus progeny contains antigenic regions of two DEN serotypes and elicits neutralizing antibodies against both.

#### Transfection and Production of Progeny Virus

Plasmid YF5'3'IV/DEN(prME) and YFM5.21DEN(E'-E) were cut with SphI and AatII restriction enzymes, appropriate YF and dengue fragments were isolated and ligated in vitro (FIG. 15) using T4 DNA ligase. After digestion with XhoI to allow run-off transcription, RNA was transcribed (using 50 ng of purified template) from the SP6 promoter and its integrity was verified by non-denaturing agarose gel electrophoresis. Vero cells were transfected with YF/DEN-2 RNA using Lipofectin (Gibco/BRL), virus was recovered from the supernatants, amplified twice in Vero cells, and titrated in a standard plaque assay on Vero cells. The virus titer was  $2 \times 10^6$  PFU/ml.

#### Nucleotide Sequencing of YF/DEN-2 Chimera

Vero cells were infected with YF/DEN-2 (clone 5.75) at an MOI of 0.1. After 96 hours, cells were harvested with Trizol (Life Technologies, Inc.). Total RNA was primed with a YF-5' end NS1 minus oligo, and reverse transcribed with Superscript II RT following a long-RT protocol (Life Technologies, Inc.). Amplification of cDNA was achieved with XL-PCR kit (Perkin Elmer). Several primers specific for dengue type 2 strain PUO-218 were used in individual sequencing reactions and standard protocols for cycle sequencing were performed. Sequence homology comparisons were against the PUO-218 strain prME sequence (GenBank accession number D00345).

Sequencing showed that the YF/DEN-2 chimera prME sequence is identical to that of PUO-218 (Gruenberg et al., J. Gen. Virol 69:1391-1398, 1988). In addition, a NarI site was introduced at the 3' end of E, resulting in amino acid change Q494G (this residue is located in the transmembrane domain and not compared in Table 21). In Table 21, amino acid differences in the prME region of YF/DEN2 is compared with prototype New Guinea C (NGC) virus and the attenuated dengue-2 vaccine strain PR-159 S1 (Hahn et al., Virology 162:167-180, 1988).

#### Growth Kinetics in Cell Culture

The growth kinetics of the YF/DEN-2 chimera were compared in Vero and FeRhL cells (FIG. 16). Cells were grown to confluency in tissue culture flask (T-75). FeRhL cells were grown in MEM containing Earle's salt, L-Glu, non-essential amino acids, 10% FBS and buffered with sodium bicarbonate, and Vero cells were grown in MEM-Alpha, L-Glu, 10% FBS (both media purchased from Gibco/BRL). Cells were inoculated with YF/DEN2 at 0.1 MOI. After 1 hour of incubation at 37° C., medium containing 3% FBS was added, and flasks were returned to a CO<sub>2</sub> incubator. Every 24 hours, aliquots of 0.5 ml were removed, FBS was added to a final concentration of 20%, and frozen for determination of titers in a plaque assay. Forty eight hours post infection CPE was observed in FeRhL cells and reached 100% by day 3. In Vero cells, CPE was less dramatic and did not reach 100% by the completion of the experiment (day 5). As shown, the YF/DEN2 reached its maximum titer ( $7.4 \log_{10}$  pfu/ml) by day 3 and lost about one log ( $6.4 \log_{10}$  pfu/ml) upon further incubation at 37° C., apparently due to death of host cells and virus degradation at this temperature. The maximum virus titer in Vero cells was achieved by day

2 ( $7.2 \log_{10}$  pfu/ml) and only half log virus ( $6.8 \log_{10}$  pfu/ml) was lost on the following 3 days. This higher rate of viable viruses in Vero cells may be explained by incomplete CPE observed in these cells. In sum, the chimera grows well in approved cell substrate for human use.

#### Neurovirulence Phenotype in Suckling Mice

Although wild-type unpassaged dengue viruses replicate in brains of suckling mice and hamsters inoculated by the intracerebral route (Brandt et al., J. Virol 6:500-506, 1970), they usually induce subclinical infection and death occur only in rare cases. However, neurovirulence for mice can be achieved by extensive passage in mouse brain. Such neuroadapted viruses can be attenuated for humans. For example, the New Guinea C (NGC), the prototype dengue 2 virus isolated in 1944 and introduced into the Americas in 1981, is not neurovirulent for suckling mice; however after sequential passage in mouse brain it became neurovirulent for mice, but was attenuated for humans (Sabin, Am. J. Trop. Med. Hyg., 1:30-50, 1952; Sabin et al., Science 101:640-642, 1945; Wisseman et al., Am. J. Trop. Med. Hyg. 12:620-623, 1963). The PUO-218 strain is a wild type dengue 2 virus isolated in 1980 epidemic in Bangkok. It is closely related to the NGC strain by nucleotide sequencing (Gruenberg et al., J. Gen. Virol 69:1391-1398, 1988). When the prME genes of the PUO-218 strain were inserted into the neuroadapted NGC backbone, the chimeric virus was attenuated for 3-days old mice inoculated by the I.C. route (Peter Wright, X<sup>th</sup> International Congress of Virology, Jerusalem, Israel, 1996). The PUO218 virus differs from NGC in one amino acid in prM (residue 55 is F in NGC and is L in PUO218) and 6 amino acids in the E protein (71 D->E, 126K->E, 1411->V, 164 I->V, 4021->F, and 484 V->I) (see Table 21). All amino acid differences (except residue E-126) are also present in PR S1 strain (attenuated vaccine strain), indicating that they may not be involved in attenuation. Only residue 126 on the E protein is different between these viruses. This residue was shown to be responsible for the neurovirulent phenotype of the mouse adapted NGC (Bray et al., J. Virology 72:1647-1651, 1998). Although mouse neurovirulence does not predict virulence/attenuation of dengue viruses for humans, it is important to determine the neurovirulence of a YF/DEN-2 chimeric virus. YF 17D retains a degree of neurotropism for mice, and causes (generally subclinical) encephalitis in monkeys after IC inoculation. For vaccine development of a den/YF chimera it will be necessary to show that the construct does not exceed YF 17D in neuroinvasiveness and neurovirulence. Ultimately safety studies in monkeys will be required. In initial studies, we determined if insertion of the prME of the PUO218 into YF 17D vaccine strain will affect its neurovirulence for suckling mice (Table 24). Groups of 3, 5, 7, and 9 days old suckling mice were inoculated by the I.C. route with 10,000 pfu of YF/DEN-2 or YF/JE<sub>SA14-14-2</sub> chimera and observed for paralysis or death for 21 days. For controls similar age groups were inoculated either sham with medium (I.C. or I.P.) or with 1,000 pfu of unpassaged commercial YF vaccine (YF-VAX® (Yellow Fever 17D vaccine)) by the I.P. route (it is not necessary to inoculate suckling mice with YF-VAX® (Yellow Fever 17D vaccine) by the I.C. route because we have previously shown that this vaccine is virulent for 4 weeks old mice by this route).

As shown in FIG. 17, all suckling mice (3 to 7 days old) inoculated by the I.C. route with the YF/DEN2 chimera died between 11 and 14 days post inoculation, whereas 8 out of 10 suckling mice (9 days old) survived. Similarly, all suckling mice (3-5 days old) inoculated with YF-VAX® (Yellow Fever 17D vaccine) by the I.P. route, with a dose which was

10-fold lower than the YF/Den2 chimera, died between 11 to 13 days post inoculation. All nine day old, as well as 8 out of 9 seven day old, mice inoculated with the YF-VAX® (Yellow Fever 17D vaccine) survived. Similar results to the YF/Den2 chimera obtained with suckling mice inoculated with the YF/JE<sub>SA14-14-2</sub> chimera.

As is mentioned above, when prME genes of the PUO218 strain were inserted into the NGC backbone the chimeric virus was not neurovirulent for 3 days old suckling mice inoculated by the I.C. route. In contrast, when these genes were inserted into the 17D backbone, the resulting YF/Den2 chimera demonstrated a neurovirulence phenotype (for suckling mice) similar to the YF/JE<sub>SA14-14-2</sub>. This experiment also demonstrated that the replacement of the prME genes of the YF 17 D with prME genes of the Dengue 2 PUO218 resulted in a chimeric virus which was less neurovirulent than the 17D parent strain.

Unlike most flaviviruses, there is no correlation between neurovirulence of dengue viruses in mice and humans. Currently the most suitable animal models for dengue infection are Old World monkeys, New World monkeys, and apes that develop subclinical infection and viremia. There is, however, no animal model for the most severe illness (DHF) in humans, which occurs when individuals become infected with a heterologous serotype due to antibody dependent enhancement of infection. Today it is generally accepted that a tetravalent vaccine is required to induce protective immunity in human beings against all four serotypes to avoid sensitizing vaccinee to more severe illness DHF. For the last fifty years, many approaches have been undertaken to produce effective dengue vaccines and although dengue viruses have been satisfactory attenuated (e.g., PR-159/S-1 for Dengue 2) in many cases in vitro or in vivo correlation of attenuation were not reproducible in humans. A current strategy is to test selected live virus vaccine candidates stepwise in small numbers of human volunteers. Many laboratories around the world are exploring various strategies to produce suitable vaccine candidates. These range from subunit vaccines including prME (protein vaccine or DNA vaccine) of dengue viruses to live attenuated whole viruses (produced by tissue culture passage or recombinant DNA technology). Although some of these candidates have shown promise in preclinical and human volunteers, development of a successful dengue vaccine remained to be implemented.

Evaluating the immunogenicity and protective efficacy of the YF/Den2 chimera in monkeys should shed light on selection of appropriate prME genes (form wild type or attenuated strain) for construction of all 4 serotypes of chimeric dengue viruses.

#### Construction of Chimeric Templates for Other Flaviviruses

Procedures for generating full-length cDNA templates encoding chimeric YF/MVE, YF/SLE, YF/WN, and YF/TBE viruses are similar to those described above for the YF/DEN-2 system. Table 20 illustrates the features of the strategy for generating YF 17D-based chimeric viruses. The unique restriction sites used for in vitro ligation, and the chimeric primers for engineering the C/prM and E/NS1 junctions are also shown. Sources of cDNA for these heterologous flaviviruses are readily available (MVE: Dalgarno et al., J. Mol. Biol. 187:309-323, 1986; SLE: Trent et al., Virology 156:293-304, 1987; TBE: Mandl et al., Virology 166:197-205, 1988; Dengue 1: Mason et al., Virology 161:262-267, 1987; Dengue 2: Deubel et al., Virology 155:365-377, 1986; Dengue 3: Hahn et al., Virology 162:167-180, 1988; Dengue 4: Zhao et al., Virology 155:77-88, 1986).

An alternative approach to engineering additional chimeric viruses is to create the C/prM junction by blunt end ligation of PCR-derived restriction fragments having ends that meet at this junction and 5' and 3' termini that flank appropriate restriction sites for introduction into YF5'3'IV or an intermediate plasmid such as pBS-KS(+). The option to use a chimeric oligonucleotide or blunt-end ligation will vary, depending on the availability of unique restriction sites within the envelope protein coding region of the virus in question.

#### Construction of YF Viruses Encoding HCV Antigens

Because the structural proteins E1 and E2 of HCV are not homologous to the structural proteins of the flaviviruses described above, the strategy for expression of these proteins involves insertion within a nonessential region of the genome, such that all of these proteins are then co-expressed with yellow fever proteins during viral replication in infected cells. The region to be targeted for insertion of the proteins is the N terminal portion of the NS1 protein, since the entire NS1 protein is not required for viral replication. Because of the potential problems with stability of the YF genome in the presence of heterologous sequence exceeding the normal size of the genome (approximately 10,000 nucleotides), the detection strategy described below can be used. In addition, deletion of NS1 may be advantageous in the chimeric YF/Flavivirus systems described above, because partial deletion of this protein may abrogate the immunity to YF associated with antibodies against NS1, and thus avoid problems with vector immunity if more than one chimeric vaccine was to be needed in a given recipient, or if a YF vaccine had been previously given or needed at a future point.

The strategy involves creating a series of in-frame deletions within the NS1 coding region of the YFM5.2 plasmid, in conjunction with engineering a translational termination codon at the end of E, and a series of two IRESs (internal ribosome entry sites). One IRES is immediately downstream of the termination codon and allows for expression of an open reading frame within the region between E and NS1. The second IRES initiates translation from truncated NS1 proteins, providing expression of the remainder of the YF nonstructural polyprotein. These derivatives are tested for recovery of infectious virus and the construct with the largest deletion is used for insertion of foreign sequences (e.g., HCV proteins) in the first IRES. This particular construct can also serve as a basis for determining whether deletion of NS1 will affect vector-specific immunity in the context of YF/Flavivirus chimeric viruses expressing prME, as described above.

The insertion of nucleotides encoding E1, E2, and/or E1 plus E2 HCV proteins is limited by the size of the deletion tolerated in the NS 1 protein. Because of this, truncated HCV proteins can be used to enhance stability within the modified YF clone. The HCV proteins are engineered with an N-terminal signal sequence immediately following the IRES and a termination codon at the C terminus. This construction will direct the HCV proteins into the endoplasmic reticulum for secretion from the cell. The strategy for this construction is shown schematically in FIG. 16. Plasmids encoding HCV proteins of genotype 1 can be used for these constructions, for example, HCV plasmids obtained from Dr. Charles Rice at Washington University (Grakoui et al., J. Virology 67:1385-1395, 1993), who has expressed this region of the virus in processing systems and within a replication-complement full-length HCV clone.

### PrM Cleavage Deletion Mutants as Attenuating Vaccine Candidates for Flaviviruses

Additional chimeric viruses included in the invention contain mutations that prevent prM cleavage, such as mutations in the prM cleavage site. For example, the prM cleavage site in flavivirus infectious clones of interest, such as dengue, TBE, SLE, and others can be mutated by site-directed mutagenesis. Any or all of the amino acids in the cleavage site, as set forth above, can be deleted or substituted. A nucleic acid fragment containing the mutated prM-E genes can then be inserted into a yellow fever virus vector using the methods described above. The prM deletion can be used with or without other attenuating mutations, for example, mutations in the E protein, to be inserted into the yellow fever virus. These mutants have advantages over single substitution mutants as vaccine candidates, because it is almost impossible to revert the deleted sequence and restore virulence.

The following chimeric flaviviruses of the invention were deposited with the American Type Culture Collection (ATCC) in Rockville, Md., U.S.A. under the terms of the Budapest treaty and granted a deposit date of Jan. 6, 1998: Chimeric Yellow Fever 17D/Dengue Type 2 Virus (YF/DEN-2; ATCC accession number ATCC VR-2593) and Chimeric Yellow Fever 17D/Japanese Encephalitis SA<sub>14</sub>-14-2 Virus (YF/JE A1.3; ATCC accession number ATCC VR-2594).

TABLE 1

Sequence comparison of JE strains and YF/JE chimeras												
Virus	E 107	E 138	E 176	E 177	E 227	E 243	E 244	E 264	E 279	E 315		
JE SA <sub>14</sub> -14-2	F	K	V	T	S	K	G	H	M	V		
YF/JE SA <sub>14</sub> -14-2	F	K	V	A	S	E	G	H	M	V		
YF/JE Nakayama	L	E	I	T	P	E	E	Q	K	A		
JE Nakayama	L	E	I	T	P	E	E	Q	K	A		
JE SA14	L	E	I	T	S	E	G	Q	K	V		

TABLE 2

Characterization of YF/JE chimeras					
Clone	Yield (μg)	Infectivity plaques/100 ng LLC-MK2	PBS log titer VERO	RNase log titer VERO	DNase log titer VERO
YF5.21v	5.5	15	7.2	0	7
YF/JE-S	7.6	50	6.2	0	6.2
YF/JE-N	7	60	5	0	5.4

TABLE 3

Plaque reduction neutralization titers on YF/JE chimeras					
Virus	non-immune ascitic fluid	YF ascitic fluid	JE ascitic fluid	non-immune IgG	YF IgG
YF5.2iv	<1.3	3.7	<1.3	<2.2	>4.3
JE SA <sub>14</sub> -14-2	<1.3	<1.3	3.4	<2.2	<2.2
YF/JE SA <sub>14</sub> -14-2	<1.3	<1.3	3.1	<2.2	<1.9
YF/JE Nakayama	<1.3	<1.3	3.4	<2.2	<2.2

TABLE 4

Neurovirulence of YF/JE SA <sub>14</sub> -14-2 Chimera 3 week old male ICR mice		
	log dose I.C.	% Mortality
YF5.2iv	4	100 (7/7)
YF/JE SA <sub>14</sub> -14-2	4	0 (0/7)
YF/JE SA <sub>14</sub> -14-2	5	0 (0/7)
YF/JE SA <sub>14</sub> -14-2	6	0 (0/8)

TABLE 5

Neuroinvasiveness of YF/JE Chimeras 3 week old male ICR mice		
	log dose (intraperitoneal)	% mortality
YF/JE Nakayama	4	0 (0/5)
YF/JE Nakayama	5	0 (0/4)
YF/JE Nakayama	6	0 (0/4)
YF/JE SA <sub>14</sub> -14-2	4	0 (0/5)
YF/JE SA <sub>14</sub> -14-2	5	0 (0/4)
YF/JE SA <sub>14</sub> -14-2	6	0 (0/4)

TABLE 6

Doses and routes of virus inoculation into groups of 4-week-old ICR mice					
Group	YF/JE s.c. log <sub>10</sub> pfu	YF/JE i.c. log <sub>10</sub> pfu	YF-VAX ® (Yellow Fever 17D vaccine)	YF-VAX ® (Yellow Fever 17D vaccine)	Total # mice
			s.c. log <sub>10</sub> pfu	i.c. log <sub>10</sub> pfu	
1	5	4.5	4.7	4.2	20
2	4	4	4.4	3.9	20
3	3	3	3.4	3.4	20
4	2	2	2.4	2.4	20
5	1	1	1.4	1.4	20
6	JE-VAX ® (inactivated Japanese Encephalitis virus vaccine) (BIKEN) 1:30, day 0, 7, s.c.				5
7	JE-VAX ® (inactivated Japanese Encephalitis virus vaccine) (BIKEN) 1:300, day 0, 7, s.c.				5
8	control s.c. (medium + 10% FBS)				5
9	control i.c. (medium + 10% FBS)				5

TABLE 7

Geometric mean neutralizing antibody titers 3 and 8 weeks after immunization, outbred mice inoculated with graded doses of vaccines by the s.c. route					
Vaccine	Dose log <sub>10</sub> PFU	Antibody titer (GMT ± SD) vs.			
		JE		YF 17D	
		3w	8w	3w	8w
YF/JE	5.0	151 ± 93	5,614 ± 3514		
	4.0	38 ± 60	127 ± 247		
	3.0	19 ± 65	43 ± 560		
	2.0	7 ± 12	3 ± 71		
	1.0	2 ± 8	0		
YF 17D	4.7			2 ± 4	18 ± 13
	4.4			35 ± 24	250 ± 109
	3.4			9 ± 20	54 ± 179
	2.4			1 ± 0	53 ± 22
	1.4			0	46 ± 18

TABLE 8

Immunogenicity and protection vs. challenge Mice were immunized on Day 0 with live vaccines and on days 0, 7, and 20 with JE-VAX ® (inactivated Japanese Encephalitis virus vaccine), bled on day 21 and challenged on day 28.					
Virus	No./ group	Dose (pfu)	Route	Total no. mice	
1. YF/JE (SA <sub>14</sub> -14-2 RMS)*	8	10 <sup>2</sup> -10 <sup>5</sup>	sc	32	
15 2. YF 17D (iv5.2) (Vero)	8	10 <sup>2</sup> -10 <sup>5</sup>	sc	32	
3. YF 17D (PMC)	8	10 <sup>2</sup> -10 <sup>5</sup>	sc	32	
4. JE Nakayama	8	10 <sup>2</sup> -10 <sup>5</sup>	sc	32	
5. JE SA <sub>14</sub> -14-2 (BHKP1)**	8	10 <sup>2</sup> -10 <sup>5</sup>	sc	32	
6. YF/JE (Nakayama)#	8	10 <sup>2</sup> -10 <sup>5</sup>	sc	32	
20 7. JE-VAX ® (inactivated Japanese Encephalitis virus vaccine) Connaught lot EJN*151B	8	100 ul 1:300 dil. on Day 0, 7 and 100 ul 1:5 dil. on D 20	sc	8	
8. None (challenged)	8	...	ip	8	
9. None (unchallenged)	8	---	---	8	
*YF/JE SA <sub>14</sub> -14-2 vaccine candidate					
**Chinese live vaccine, passed once in BHK cells					
#Chimeric YF/JE virus, with prM-E insert of wild-type JE Nakayama					

TABLE 9

Protection of C57/BL6 mice by a single SC inoculum of graded doses of live  
virus vaccines against IP challenge with 158 LD<sub>50</sub> of wild-type JE virus (IC-37).  
Mice were challenged 28 days after immunization.

Vaccine	Number of survivors/number challenged (% survivors) by vaccine dose (log <sub>10</sub> pfu)						
	None	1	2	3	4	5	Other
Yellow fever 17D (YF-VAX ® (Yellow Fever 17D vaccine) unpassaged)	NT*	3/8 (37.5%)	1/8 (12.5%)	1/8 (12.5%)	2/8 (25%)		
Yellow fever 17D (YF5.2iv infectious clone)	NT	0/8 (0%)	1/8 (12.5%)	1/8 (12.5%)	1/8 (12.5%)		
Yellow fever/JE SA14-14-2 chimera	1/8 (12.5%)	2/8 (25%)	7/7 (100%)	7/8 (87.5%)	7/7 (100%)		
Chinese JE vaccine SA14-14-2 (BHK1)	NT	1/8 (12.5%)	1/8 (12.5%)	0/8 (0%)	3/8 (37.5%)		
Wild-Type JE (Nakayama)#	NT	2/7 (29%)	1/6 (17%)	1/3 (33%)	1/4 (25%)		
YF/JE (Nakayama)		3/3 (100%)	5/5 (100%)	3/3 (100%)			
Mouse brain vaccine (JE-VAX ® (inactivated Japanese Encephalitis virus vaccine))**							7/8 (87.5%)
Control (challenge)	1/8 (12.5%)						
Control (no challenge)	8/8 (100%)						

\*Not tested

#Some mice died as a result of inoculation of the wild-type virus at high doses, thus fewer mice remained for challenge

\*\*Three doses at 1 week intervals

\*No mice survived initial inoculation at this dose

TABLE 10

Geometric mean neutralizing antibody titers, C57/BL6 mice 21 days after immunization with a single SC inoculum of graded doses of live virus vaccines and 1 day after the third dose of inactivated JE-VAX ® (inactivated Japanese Encephalitis virus vaccine).				
Vaccine	Dose (log <sub>10</sub> PFU)	Antibody titer (GMT ± SD) vs.		
		JEV	YF 17D	
YF/JE SA <sub>14</sub> -14-2	5	44.8 ± 25.2		
	4	26.5 ± 23.1		
	3	6.2 ± 4.9		
	2	1.1 ± 0.35		
SA <sub>14</sub> -14-2 (BHK1)	1	1 ± 0		
	5	2.5 ± 4.3		
	4	3.5 ± 20.5		
	3	4.7 ± 15.5		
JE Nakayama	2	1 ± 0		
	5	1.32 ± 1		
	4	4 ± 4.0		
	3	1.6 ± 1.8		
YF/JE SA <sub>14</sub> -14-2	2	1 ± 0		
	5	10 ± 70*		
	4	102.5 ± 45.7		
	3	76.8 ± 63.9		
JE-VAX ® (inactivated Japanese Encephalitis virus (mouse brain))	2	19.8 ± 8.1		
	3 doses**	2.8 ± 6.5		
YF-VAX ® (Yellow Fever 17D vaccine)	5		11 ± 9.6	
	4		13.8 ± 19.1	
	3		4.3 ± 11.7	
	2		1 ± 0	
YF5.2iv (17D infect. clone)	5		29.3 ± 47.1	
	4		11 ± 15.2	
	3		8 ± 19.4	
	2		2.1 ± 3.2	
Controls	0	1 ± 0		

\*only 2/8 mice survived immunization with this virus; the low antibody titers in these animals probably reflect low level virus replication consistent with survival.

\*\*3 doses on days 0, 7, and 20; animals were bled on day 21, 1 day after their third immunization. The day 20 boost was performed with a higher dose of vaccine, thus antibody titers pre-challenge are expected to be higher than those shown here.

TABLE 11

Geometric mean neutralizing antibody titers (GMT) in 3 monkeys 2 and 4 weeks post inoculation with a single dose of YF-VAX ® (Yellow Fever 17D vaccine) or CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) by the I.C. route				
Vaccine	Dose (log <sub>10</sub> pfu)	GMT		
		JE	YF	
CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins)	7.0	75	3200	
YF-VAX ® (Yellow Fever 17D vaccine)	5.0		66	4971

TABLE 12

Immunization and protection: rhesus monkeys Screening HI test for flavivirus antibodies: negative				
Group	N	Virus	Dose, route (log <sub>10</sub> PFU/0.5 ml)	JE Challenge Day 60
1	3	YF/JE SA <sub>14</sub> -14-2	4.3 SC	5.0 IC
2	3	YF/JE SA <sub>14</sub> -14-2	5.3 SC	5.0 IC
3	4	Saline/sham	— SC	5.0 IC

15 Viremia days 1-7 after immunization and challenge  
Neutralization test days 0, 15, 30, 45, and 60 after immunization and days 15 and 30 after challenge  
Necropsy day 30 post challenge

20

TABLE 13

Viremia, rhesus monkeys immunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) by the SC route								
Monkey	log <sub>10</sub> PFU	Day post-inoculation						
		0	1	2	3	4	5	6
30 R423	4.3	<1.0*	<1.0	<1.0	1.1	1.7	1.0	<1.0
R073		<1.0	<1.0	<1.0	1.0	1.0	<1.0	<1.0
R364		<1.0	1.0	<1.0	1.0	1.0	<1.0	<1.0
R756	5.3	<1.0	1.0	1.0	1.6	1.0	<1.0	<1.0
R174		<1.0	1.3	1.8	1.6	1.1	<1.0	<1.0
R147		<1.0	2.0	1.6	1.0	1.0	<1.0	<1.0
35	*log <sub>10</sub> PFU/ml							

TABLE 14

JE neutralizing antibody responses, rhesus monkeys immunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) by the SC Route 50% PRNT titers, heat-inactivated serum, no added complement				
Monkey	log <sub>10</sub> PFU	Day post-inoculation		
		Baseline	15	30
R423	4.3	<10	160	2560
R073		<10	80	640
R364		<10	160	320
R756	5.3	<10	20	320
R174		<10	640	2560
R147		<10	160	2560

55

TABLE 15

Protection against IC challenge, rhesus monkeys immunized with CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins) by the SC route Monkeys challenged IC on Day 60 with 100,000 pfu/mouse LD50		
Vaccine Dose log <sub>10</sub> PFU	No. survived/ No. tested	
4.3	2/3 (67%)	
5.3	3/3 (100%)	
Sham	0/4 (0%)	

60

65

\*1 monkey that died was a pregnant female



TABLE 16

List of chimeric YF/JE mutants (1 to 9)  
constructed to identify residues involved in attenuation of the CHIMERIVAX™-JE  
(chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E protein).  
Mutated amino acids on the E-proteins are shown in bold letters.

Positions	Nakayama	ChimeriVax™*	Mutant Viruses										
			1	2	3	4	5	6	7	8	9	10	11
107	L	F	L	F	F	L	L	F	L	F	L	F	L
138	E	K	K	E	K	K	E	E	E	E	E	E	E
176	I	V	V	V	I	I	V	I	I	V	V	I	I
177	T	A	A	A	T	T	A	T	T	A	A	T	T
227	P	S	S	S	S	S	S	S	P	P	P	P	P
264	Q	H	H	H	H	H	H	H	Q	Q	Q	Q	Q
279	K	M	M	M	M	M	M	M	K	K	K	K	K

\*CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins)

20

TABLE 17

Group	Dose administered i.c. (pfu)		
	P1	P10	P18
Neat	$\geq 6 \times 10^4$	$1 \times 10^6$	$2 \times 10^7$
$10^{-1}$	$\geq 6 \times 10^3$	$1 \times 10^5$	$2 \times 10^6$

25

30

TABLE 18

Group	Dose administered s.c. (pfu)		
	RMS	P10	P18
Neat	$2 \times 10^5$	$2 \times 10^7$	$3 \times 10^7$
$10^5$	$1 \times 10^5$	$5 \times 10^5$	$5 \times 10^4$
$10^4$	$1 \times 10^4$	$5 \times 10^4$	$5 \times 10^3$

TABLE 19

Design of an experiment to determine cross-protection/interference between YF 17D and YF/JE SA <sub>14</sub> -14-2				
# of female		2 <sup>nd</sup> vaccine		
ICR mice	1 <sup>st</sup> Vaccine <sup>a</sup>	3 months	6 months	12 months
8	YF/JE SA <sub>14</sub> -14-2	YF-VAX ® (Yellow Fever 17D vaccine)		
8	YF/JE SA <sub>14</sub> -14-2		YF-VAX ® (Yellow Fever 17D vaccine)	
8	YF/JE SA <sub>14</sub> -14-2			YF-VAX ® (Yellow Fever 17D vaccine)
8	JE-VAX ® (inactivated Japanese Encephalitis virus vaccine)	YF-VAX ® (Yellow Fever 17D vaccine)		
8	JE-VAX ® (inactivated Japanese Encephalitis virus vaccine)		YF-VAX ® (Yellow Fever 17D vaccine)	
8	JE-VAX ® (inactivated Japanese Encephalitis virus vaccine)			YF-VAX ® (Yellow Fever 17D vaccine)
8	YF-VAX ® (Yellow Fever 17D vaccine)	YF/JE SA <sub>14</sub> -14-2		
8	YF-VAX ® (Yellow Fever 17D vaccine)		YF/JE SA <sub>14</sub> -14-2	
8	YF-VAX ® (Yellow Fever 17D vaccine)			YF/JE SA <sub>14</sub> -14-2
4		YF-VAX ® (Yellow Fever 17D vaccine)		
4		YF/JE SA <sub>14</sub> -14-2		
4			YF-VAX ® (Yellow Fever 17D vaccine)	
4			YF/JE SA <sub>14</sub> -14-2	

TABLE 19-continued

Design of an experiment to determine cross-protection/interference between YF 17D and YF/JE SA <sub>14</sub> -14-2				
# of female	ICR mice	1 <sup>st</sup> Vaccine <sup>a</sup>	2 <sup>nd</sup> vaccine	
			3 months	6 months
4				12 months
4				YF-VAX ® (Yellow Fever 17D vaccine) YF/JE SA <sub>14</sub> -14-2

<sup>a</sup>One dose of YF/JE SA<sub>14</sub>-14-2, 5.3 log<sub>10</sub>pfu/mouse, sc.

One dose of YF-VAX ® (Yellow Fever 17D vaccine), 4.4 log<sub>10</sub>pfu/mouse, sc.

Two doses of JE-VAX ® (inactivated Japanese Encephalitis virus vaccine) (PMC), 0.5 ml of 1:5 dilution administered ip at 1 week intervals.

TABLE 20

Engineering of YF/Flavivirus chimeras					
Virus	Chimeric C/prM junction <sup>1</sup>	Chimeric E/NS1 junction <sup>2</sup>	5' ligation <sup>3</sup>	3' ligation <sup>4</sup>	Sites <sup>5</sup> eliminated or (created)
YF/WN	X-cactgggagagcttgaaggctc (SEQ ID NO:14)	aaagccagttgcagccgcggttaa (SEQ ID NO:15)	AatII	NsiI	
YF/DEN-1	X-aaggtagactgtgtgggctccc (SEQ ID NO:16)	gatactcagttaccacccgcggttaa (SEQ ID NO:17)	AatII	SphI	SphI in DEN
YF/DEN-2	X-aaggtagattgtgtgcattg (SEQ ID NO:18)	aaacctcagttaccacccgcggttaa (SEQ ID NO:19)	AatII	SphI	
YF/DEN-3	X-aaggtgaattgaagtgtctcta (SEQ ID NO:20)	acccccagcaaccacccgcggttaa (SEQ ID NO:21)	AatII	SphI	XhoI in DEN (SphI in DEN)
YF/DEN-4	X-aaaggaacagttgtctcta (SEQ ID NO:22)	acccgaagttgcaaccgcggttaa (SEQ ID NO:23)	AatII	NsiI	
YF/SLE	X-aacgtgatagttggatagtc (SEQ ID NO:24)	accgttggtgcacccgcggttaa (SEQ ID NO:25)	AatII	SphI	AatII in SLE
YF/MVE	X-aatttcgaaggttggaaggctc (SEQ ID NO:26)	gaccggtgtttacacccgcggttaa (SEQ ID NO:27)	AatII	AgeI	(AgeI in YF)
YF/TBE	X-tactgcgaacgacgttgccac (SEQ ID NO:28)	actgggaacctcaccgcggttaa (SEQ ID NO:29)	AatII	AgeI	(AgeI in YF)

<sup>1,2</sup>The column illustrates the oligonucleotide used to generate chimeric YF/Flavivirus primers corresponding to the C/prM or E/NS1 junction. (See text). X = carboxyl terminal coding sequence of the YF capsid. The underlined region corresponds to the targeted heterologous sequence immediately upstream of the NarI site (antisense = ccgggg). This site allows insertion of PCR products into the Yfm5.2 (NarI) plasmid required for generating full-length cDNA templates. Other nucleotides are specific to the heterologous virus. Oligonucleotide primers are listed 5' to 3'.

<sup>3,4</sup>The unique restriction sites used for creating restriction fragments that can be isolated and ligated in vitro to produce full-length chimeric cDNA templates are listed. Because some sequences do not contain convenient sites, engineering of appropriate sites is required in some cases (footnote 5).

<sup>5</sup>In parentheses are the restriction enzyme sites that must be created either in the YF backbone or the heterologous virus to allow efficient in vitro ligation. Sites not in parentheses must be eliminated. All such modifications are done by silent mutagenesis of the cDNA for the respective clone. Blank spaces indicate that no modification of the cDNA clones is required.

TABLE 21

Sequence comparison of Dengue-2 and YF/Den-2 <sub>218</sub> viruses							
Virus	PrM						
	28	31	55	57	125	152	161
YF/D2 <sub>218</sub>	E	V	L	R	I	A	V
PUO-218	E	V	L	R	I	A	V
NGC	E	V	F	R	T	A	V
PR-159(S1)	K	T	F	K	T	V	I

ENVELOPE													
Virus	71	81	126	129	139	141	162	164	202	203	335	352	390
YF/D2 <sub>218</sub>	E	S	E	V	I	V	I	V	E	N	I	I	N
PUO-218	E	S	E	V	I	V	I	V	E	N	I	I	N
NGC	D	S	K	V	I	I	I	I	E	N	I	I	N
PR-159(S1)	D	T	E	I	V	I	V	I	K	D	T	T	D

TABLE 22

Summary of histopathology results, monkeys inoculated with YF-VAX ® (Yellow Fever 17D vaccine) or YF/JE SA14-14-2 by the IC route					
YF-VAX ® (Yellow Fever 17D vaccine)			CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis prM and E proteins)		
Monkey No.	Discriminator area score	Discriminator plus target area score	Monkey No.	Discriminator area score	Discriminator plus target area score
N030	0.21	0.64	N191	0	0.17
N492	0.04	0.36	N290	0.09	0.06
N479	0	0.17	N431	0.13	0.09
Group means	0.08	0.39		0.07	0.11

TABLE 23

List of initial chimeric YF/JE mutants constructed to identify residues involved in attenuation of the CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus prM and E proteins). Reverted amino acids on the E-proteins are shown in BOLD												
CHIMERIVAX™-JE (chimeric flavivirus vaccine comprising Japanese Encephalitis virus			MUTANT VIRUSES									
Positions on												
E-Protein	Nakayama	prM and E proteins)	1	2	3	4	5	6	7	8	9	10
107	L	F	L	F	F	L	L	F	L	F	L	F
138	E	K	K	E	K	K	E	E	E	E	E	E
176	I	V	V	V	I	I	V	I	I	V	V	I
177	T	A	A	A	T	T	A	T	T	A	A	T
227	P	S	S	S	S	S	S	S	S	P	P	P
264	Q	H	H	H	H	H	H	H	H	Q	Q	Q
279	K	M	M	M	M	M	M	M	M	K	K	K

TABLE 24

Experiment to determine neurovirulence and neuroinvasiveness phenotypes of vaccine candidates in suckling mice					
AGE OF MICE (DAYS)					
Virus	Route	3	5	7	9
YF/Den-2	LC.	10 <sup>4</sup> *	10 <sup>4</sup>	10 <sup>4</sup>	10 <sup>4</sup>
YF/JESA14-14-2	LC.	10 <sup>4</sup>	10 <sup>4</sup>	10 <sup>4</sup>	10 <sup>4</sup>

TABLE 24-continued

Experiment to determine neurovirulence and neuroinvasiveness phenotypes of vaccine candidates in suckling mice					
AGE OF MICE (DAYS)					
Virus	Route	3	5	7	9
YF 17D	LP.	10 <sup>3</sup>	10 <sup>3</sup>	10 <sup>3</sup>	10 <sup>3</sup>
MED + 5% FBS	LC., LP.	—	—	—	—

\*PFU/0.02 ml of inoculum

TABLE 25

Summary of differences between virulent (Asibi) and attenuated (17D, 17DD, RMS, and P18) yellow fever viruses									
Gene	NT	Asibi	17D204US	RMS	P18	17D204F	17D213	17DD	AA
C	304	G	A	A	A	A	A	A	
	370	T	C	C	C	C	C	C	
non-M	643	A	A	—	—	A	A	G	
M	854	C	T	—	—	T	T	T	LF
	883	A	G	—	—	G	G	A	
E	1127	G	A	—	—	A	A	A	GR
	1140	C	T	—	—	T	T	C	AV

TABLE 25-continued

Summary of differences between virulent (Asibi) and attenuated (17D, 17DD, RMS, and P18) yellow fever viruses									
Gene	NT	Asibi	17D204US	RMS	P18	17D204F	17D213	17DD	AA
NSI	1431	A	A	—	—	A	C	A	NT
	1436	G	G	—	—	G	G	A	DS
	1437	A	A	—	—	A	A	G	
	1482	C	T	—	—	T	T	T	AV
	1491	C	T	—	—	T	T	T	TI
	1558	C	C	—	—	C	C	A	
	1572	A	C	—	—	C	C	C	KT
	1750	C	T	—	—	T	T	T	
	1819	C	T	—	—	T	T	T	
	1870	G	A	—	—	A	A	A	MI
	1887	C	T	—	—	T	T	T	SF
	1946	C	T	—	—	T	T	C	PS
	1965	A	G	—	—	G	G	G	KR
	2110	G	G	—	—	G	G	A	
	2112	C	G	—	—	G	G	G	TR
	2142	C	A	—	—	A	A	A	PH
	2219	G	A	—	—	A	A	G	AT
	2220	C	C	—	—	C	C	T	TI
	2356	C	T	—	—	T	T	T	
	2687	C	T	T	T	T	T	T	FL
	2704	A	G	G	G	G	G	G	
	3274	G	A	A	A	A	A	A	
	3371	A	G	G	G	G	G	G	VI
	3599	T	T	T	T	T	T	C	
ns2a	3613	G	A	A	A	A	A	A	
	3637	C	C	C	C	C	C	T	
	3817	G, A	G	G	G	G	G	G	
	3860	A	G	G	G	G	G	G	VM
	3915	T, A	T	T	T	T	T	T	
	4007	A	G	G	G	G	G	G	AT
	4013	C	T	T	T	T	T	C	FL
	4022	A	G	G	G	G	G	G	AT
	4025	G	G	A	A	G	G	G	VM
	4054	C	T	T	T	T	T	C	
ns2b	4056	C	T	T	T	T	T	T	FS
	4204	C	C	C	C	C	C	T	
	4289	A	C	C	C	C	C	C	LI
	4387	A	G	G	G	G	G	G	
	4505	A	C	C	C	C	C	C	LI
	4507	T	C	C	C	C	C	C	
	4612	T	C	C	C	C	C	T	
NS3	4864	G, A	G	G	G	G	G	G	
	4873	T	G	G	G	G	G	T	
	4942	A	A	A	A	A	A	G	
	4957	C	C	C	C	C	C	T	
	4972	G	G	G	G	G	G	A	
	5115	A	A	A	A	A	A	G	QR
	5131	G, T	G	G	G	G	G	G	MM, I
	5153	A	G	G	G	G	G	A	VI
	5194	T	C	C	C	C	C	C	
	5225	A	C	C	C	C	C	C	
	5362	C	C	C	C	C	T	A	
	5431	C	T	T	T	T	T	T	
	5461	T	T	C	C	T	T	T	
NS3	5473	C	T	T	T	T	T	T	
	5641	G	A	G	G	A	G	G	
	6013	C	T	T	T	T	T	T	
	6023	G	A	A	A	A	A	A	ND
	6070	C	C	C	C	C	C	T	
	6448	G	T	T	T	T	T	T	
	6514	T	T	T	T	T	T	C	
ns4a	6529	T	C	C	C	T	T	T	
	6625	A	A	A	A	A	C	C	
	6758	A	G	G	G	A	A	A	VI
	6829	T	C	C	C	C	C	C	
	6876	T	C	C	C	C	C	C	AV
	7171	A	G	G	G	G	G	G	MI
	7319	G	G	A	A	A	A	A	EK
	7497	T	T	T	T	T	C	T	LS
	7571	C	A	A	A	A	A	C	
	7580	T	C	C	C	C	C	C	HY
NS5	7642	T	C	C	C	C	C	C	
	7701	A	G	G	G	G	G	A	RQ
	7945	C	T	T	T	T	T	T	

TABLE 25-continued

Summary of differences between virulent (Asibi) and attenuated (17D, 17DD, RMS, and P18) yellow fever viruses									
Gene	NT	Asibi	17D204US	RMS	P18	17D204F	17D213	17DD	AA
3' NC	7975	C	C	C	C	C	C	T	
	8008	T	C	C	C	C	C	C	
	8029	T	T	T	T	T	T	C	
	8212	C	C	T	T	C	C	C	
	8581	A	A	C	C	A	A	A	
	8629	C	T	T	T	T	T	T	
	8808	A	A	A	A	A	A	G	NS
	9397	A	A	A	A	A	A	G	
	9605	A	G	G	G	A	A	A	DN
	10075	G, T	G	G	G	G	G	G	MM, I
	10142	G	A	A	A	A	A	A	KE
	10243	G	A	A	A	A	A	A	
	10285	T	C	C	C	C	C	C	
	10312	A	G	G	G	G	G	G	
	10316	T, C	T	T	T	T	T	T	SS, P
	10339	C	G	G	G	G	G	G	
	10367	T	C	C	C	C	C	C	
	10418	T	C	C	C	C	C	C	
	10454	A	G	A	A	A	A	A	
	10550	T	C	C	C	C	C	T	
	10722	G	G	G	G	A	G	G	
	10800	G	A	A	A	A	A	A	
	10847	A	C	C	C	C	C	C	

NT: nucleotide numbers are from the 5' terminus of the genome. Where clonal differences were present, both nucleotides as well as amino acids (if appropriate) are shown. If a nucleotide change results in an amino acid substitution, the amino acid (AA) is shown from left to right (e.g., from Asibi to 17D).

—: The genes for prME in RMS (YF17D/JESA14-14-2) and P18 (passage 18 of the RMS) are from JEV strain SA14-14-2, and therefore are not comparable with YFV sequences. Sequences for Asibi are taken from Hahn et al., 1987; 17D204US from Rice et al. 1985; and 17D204F from Dupuy et al. 1989. RMS and P18 are unpublished sequences (OraVax, Inc.), and 17D213 and 17DD are from Duarte dos Santos et al. 1994. Note that there is no sequence difference between RMS and passage 18. There are 6 nucleotide differences (nucleotide positions are shaded) between the published YF17D sequence and RMS shown in bold letters; changes in 5461, 5641, 8212, and 8581 are silent and do not result in amino acid substitutions. Changes in positions 4025 and 7319 result in amino acid substitutions.

#### Other Embodiments

Other embodiments are within the following claims. For example, the prM-E protein genes of other flaviviruses of medical importance can be inserted into the yellow fever vaccine virus backbone to produce vaccines against other medically important flaviviruses (see, e.g., Monath et al., "Flaviviruses," in *Virology*, Fields (ed.), Raven-Lippincott, N.Y., 1995, Volume 1, 961-1034). Examples of additional flaviviruses from which genes to be inserted into the chimeric vectors of the invention can be obtained include, e.g., Kunjin, Central European Encephalitis, Russian Spring-Summer Encephalitis, Powassan, Kyasanur Forest Disease, and Omsk Hemorrhagic Fever viruses. In addition, genes from even more distantly related viruses can be inserted into the yellow fever vaccine virus to construct novel vaccines. Vaccine Production and Use

The vaccines of the invention are administered in amounts, and by using methods, that can readily be determined by persons of ordinary skill in this art. The vaccines can be administered and formulated, for example, in the same manner as the yellow fever 17D vaccine, e.g., as a clarified suspension of infected chicken embryo tissue, or a fluid harvested from cell cultures infected with the chimeric yellow fever virus. Thus, the live, attenuated chimeric virus is formulated as a sterile aqueous solution containing between 100 and 1,000,000 infectious units (e.g., plaque-forming units or tissue culture infectious doses) in a dose volume of 0.1 to 1.0 ml, to be administered by, for example, intramuscular, subcutaneous, or intradermal routes. In

addition, because flaviviruses may be capable of infecting the human host via the mucosal routes, such as the oral route (Gresikova et al., "Tick-borne Encephalitis," In *The Arboviruses, Ecology and Epidemiology*, Monath (ed.), CRC Press, Boca Raton, Fla., 1988, Volume IV, 177-203), the vaccine virus can be administered by a mucosal route to achieve a protective immune response. The vaccine can be administered as a primary prophylactic agent in adults or children at risk of flavivirus infection. The vaccines can also be used as secondary agents for treating flavivirus-infected patients by stimulating an immune response against the flavivirus.

It may be desirable to use the yellow fever vaccine vector system for immunizing a host against one virus (for example, Japanese Encephalitis virus) and to later reimmunize the same individual against a second or third virus using a different chimeric construct. A significant advantage of the chimeric yellow fever system is that the vector will not elicit strong immunity to itself. Nor will prior immunity to yellow fever virus preclude the use of the chimeric vaccine as a vector for heterologous gene expression. These advantages are due to the removal of the portion of the yellow fever vaccine E gene that encodes neutralizing (protective) antigens to yellow fever, and replacement with another, heterologous gene that does not provide cross-protection against yellow fever. Although YF 17D virus nonstructural proteins may play a role in protection, for example, by eliciting antibodies against NS1, which is involved in complement-dependent antibody mediated lysis of infected cells (Schlesinger et al., *J. Immunology* 135:2805-2809, 1985),

or by inducing cytotoxic T cell responses to NS3 or other proteins of the virus, it is unlikely that these responses will abrogate the ability of a live virus vaccine to stimulate neutralizing antibodies. This is supported by the facts that (1) individuals who have been previously infected with JE virus respond to vaccination with YF 17D similarly to persons without previous JE infection, and (2) individuals who have previously received the YF 17D vaccine respond to revaccination with a rise in neutralizing antibody titers (Sweet et al., Am. J. Trop. Med. Hyg. 11:562-569, 1962). Thus, the chimeric vector can be used in populations that are immune to yellow fever because of prior natural infection or vaccination, and can be used repeatedly, or to immunize simultaneously or sequentially with several different constructs, including yellow fever chimeras with inserts from, for example, Japanese Encephalitis, St. Louis Encephalitis, or West Nile viruses.

For vaccine applications, adjuvants that are known to those skilled in the art can be used. Adjuvants that can be used to enhance the immunogenicity of the chimeric vaccines include, for example, liposomal formulations, synthetic adjuvants, such as saponins (e.g., QS21), muramyl dipeptide, monophosphoryl lipid A, or polyphosphazene. Although these adjuvants are typically used to enhance immune responses to inactivated vaccines, they can also be used with live vaccines. In the case of a chimeric vaccine delivered via a mucosal route, for example, orally, mucosal adjuvants such as the heat-labile toxin of *E. coli* (LT) or mutant derivations of LT are useful adjuvants. In addition, genes encoding cytokines that have adjuvant activities can be inserted into the yellow fever vectors. Thus, genes encoding cytokines, such as GM-CSF, IL-2, IL-12, IL-13, or IL-5, can be inserted together with heterologous flavivirus genes to produce a vaccine that results in enhanced immune responses, or to modulate immunity directed more specifically towards cellular, humoral, or mucosal responses.

In addition to vaccine applications, as one skilled in the art can readily understand, the vectors of the invention can be used in gene therapy methods to introduce therapeutic gene products into a patient's cells and in cancer therapy. In these methods, genes encoding therapeutic gene products

are inserted into the vectors, for example, in place of the gene encoding the prM-E protein.

Yellow fever 17D virus targets cells of the lymphoid and reticuloendothelial systems, including precursors in bone marrow, monocytes, macrophages, T cells, and B cells (Monath, "Pathobiology of the Flaviviruses," pp. 375-425, in Schlesinger & Schlesinger (Eds.), *The Togaviridae and Flaviviridae*, Plenum Press, New York 1986). The yellow fever 17D virus thus naturally targets cells involved in antigen presentation and immune stimulation. Replication of the virus in these cells, with high-level expression of heterologous genes, makes yellow fever 17D vaccine-virus an ideal vector for gene therapy or immunotherapy against cancers of the lymphoreticular system and leukemias, for example. Additional advantages are that (1) the flavivirus genome does not integrate into host cell DNA, (2) yellow fever virus appears to persist in the host for prolonged periods, and (3) that heterologous genes can be inserted at the 3' end of the yellow fever vector, as described above in the strategy for producing a Hepatitis C vaccine. Yellow fever 17D virus can be used as a vector carrying tumor antigens for induction of immune responses for cancer immunotherapy. As a second application, yellow fever 17D can be used to target lymphoreticular tumors and express heterologous genes that have anti-tumor effects, including cytokines, such as TNF-alpha. As a third application, yellow fever 17D can be used to target heterologous genes to bone marrow to direct expression of bioactive molecules required to treat hematologic diseases, such as, for example, neutropenia; an example of a bioactive molecule that can be used in such an application is GM-CSF, but other appropriate bioactive molecules can be selected by those skilled in the art.

An additional advantage of the yellow fever vector system is that flaviviruses replicate in the cytoplasm of cells, so that the virus replication strategy does not involve integration of the viral genome into the host cell (Chambers et al., "Flavivirus Genome Organization, Expression, and Replication," in *Annual Review of Microbiology* 44:649-688, 1990), providing an important safety measure.

All references cited herein are incorporated by reference in their entirety.

## SEQUENCE LISTING

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<210> SEQ ID NO 1

<211> LENGTH: 1983

<212> TYPE: DNA

<213> ORGANISM: Dengue-2 virus

<220> FEATURE:

<221> NAME/KEY: CDS

<222> LOCATION: (1)...(1983)

<400> SEQUENCE: 1

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Phe His Leu Thr Thr Arg Asn Gly Glu Pro His Met Ile Val Ser Arg	
1 5 10 15	
caa gag aaa ggg aaa agt ctt ttg ttt aaa aca gag gat ggc gtg aac	96
Gln Glu Lys Gly Lys Ser Leu Leu Phe Lys Thr Glu Asp Gly Val Asn	
20 25 30	
atg tgc acc ctc atg gcc atg gac ctt ggt gaa ttg tgt gaa gac aca	144
Met Cys Thr Leu Met Ala Met Asp Leu Gly Glu Leu Cys Glu Asp Thr	
35 40 45	

-continued

atc	acg	tac	aag	tgt	ccc	ctt	ctc	agg	cag	aat	gag	cca	gaa	gac	ata	192
Ile	Thr	Tyr	Lys	Cys	Pro	Leu	Leu	Arg	Gln	Asn	Glu	Pro	Glu	Asp	Ile	
	50					55					60					
gac	tgc	tgg	tgc	aac	tcc	acg	tcc	acg	tgg	gta	acc	tat	ggg	act	tgt	240
Asp	Cys	Trp	Cys	Asn	Ser	Thr	Ser	Thr	Trp	Val	Thr	Tyr	Gly	Thr	Cys	
	65				70				75						80	
acc	acc	acg	gga	gaa	cat	aga	aga	gaa	aaa	aga	tca	gtg	gca	ctc	gtt	288
Thr	Thr	Thr	Gly	Glu	His	Arg	Arg	Glu	Lys	Arg	Ser	Val	Ala	Leu	Val	
				85					90					95		
cca	cat	gtg	gga	atg	gga	ctg	gag	acg	cga	act	gaa	aca	tgg	atg	tca	336
Pro	His	Val	Gly	Met	Gly	Leu	Glu	Thr	Arg	Thr	Glu	Thr	Trp	Met	Ser	
			100				105						110			
tca	gaa	ggg	gct	tgg	aaa	cat	gcc	cag	aga	att	gaa	att	tgg	atc	ctg	384
Ser	Glu	Gly	Ala	Trp	Lys	His	Ala	Gln	Arg	Ile	Glu	Ile	Trp	Ile	Leu	
	115					120						125				
aga	cat	cca	ggc	ttc	acc	ata	atg	gca	gca	atc	ctg	gca	tac	acc	ata	432
Arg	His	Pro	Gly	Phe	Thr	Ile	Met	Ala	Ala	Ile	Leu	Ala	Tyr	Thr	Ile	
	130				135					140						
ggg	acg	aca	cat	ttc	cag	aga	gca	ctg	att	ttc	atc	tta	ctg	aca	gct	480
Gly	Thr	Thr	His	Phe	Gln	Arg	Ala	Leu	Ile	Phe	Ile	Leu	Leu	Thr	Ala	
	145				150				155						160	
gtc	gct	cct	tca	atg	aca	atg	cgt	tgc	ata	gga	ata	tca	aat	aga	gac	528
Val	Ala	Pro	Ser	Met	Thr	Met	Arg	Cys	Ile	Gly	Ile	Ser	Asn	Arg	Asp	
			165					170					175			
ttt	gta	gaa	ggg	gtt	tca	gga	gga	agc	tgg	gtt	gac	ata	gtc	tta	gaa	576
Phe	Val	Glu	Gly	Val	Ser	Gly	Gly	Ser	Trp	Val	Asp	Ile	Val	Leu	Glu	
		180					185						190			
cat	gga	agc	tgt	gtg	acg	acg	atg	gca	aaa	aac	aaa	cca	aca	ttg	gat	624
His	Gly	Ser	Cys	Val	Thr	Thr	Met	Ala	Lys	Asn	Lys	Pro	Thr	Leu	Asp	
	195					200						205				
ttt	gaa	ctg	ata	aaa	aca	gaa	gcc	aaa	cag	cct	gcc	acc	cta	agg	aag	672
Phe	Glu	Leu	Ile	Lys	Thr	Glu	Ala	Lys	Gln	Pro	Ala	Thr	Leu	Arg	Lys	
	210				215						220					
tac	tgt	ata	gag	gca	aag	cta	acc	aac	aca	aca	aca	gaa	tct	cgt	tgc	720
Tyr	Cys	Ile	Glu	Ala	Lys	Leu	Thr	Asn	Thr	Thr	Thr	Glu	Ser	Arg	Cys	
	225				230				235						240	
cca	aca	caa	ggg	gaa	ccc	agc	cta	aat	gaa	gag	cag	gat	aaa	agg	ttc	768
Pro	Thr	Gln	Gly	Glu	Pro	Ser	Leu	Asn	Glu	Glu	Gln	Asp	Lys	Arg	Phe	
			245					250					255			
gtc	tgc	aaa	cac	tcc	atg	gta	gac	aga	gga	tgg	gga	aat	gga	tgt	gga	816
Val	Cys	Lys	His	Ser	Met	Val	Asp	Arg	Gly	Trp	Gly	Asn	Gly	Cys	Gly	
		260					265					270				
tta	ttt	gga	aag	gga	ggc	att	gtg	acc	tgt	gct	atg	ttc	aca	tgc	aaa	864
Leu	Phe	Gly	Lys	Gly	Gly	Ile	Val	Thr	Cys	Ala	Met	Phe	Thr	Cys	Lys	
	275					280						285				
aag	aac	atg	gag	gga	aaa	gtt	gtg	cag	cca	gaa	aac	ttg	gaa	tac	acc	912
Lys	Asn	Met	Glu	Gly	Lys	Val	Val	Gln	Pro	Glu	Asn	Leu	Glu	Tyr	Thr	
	290				295					300						
att	gtg	gta	aca	ccc	cac	tca	ggg	gaa	gag	cat	gcg	gtc	gga	aat	gac	960
Ile	Val	Val	Thr	Pro	His	Ser	Gly	Glu	Glu	His	Ala	Val	Gly	Asn	Asp	
	305				310				315					320		
aca	gga	aaa	cat	ggc	aag	gaa	atc	aaa	gta	aca	cca	cag	agt	tcc	atc	1008
Thr	Gly	Lys	His	Gly	Lys	Glu	Ile	Lys	Val	Thr	Pro	Gln	Ser	Ser	Ile	
			325					330					335			
aca	gaa	gca	gaa	ttg	aca	ggc	tat	ggc	act	gtc	acg	atg	gag	tgc	tct	1056
Thr	Glu	Ala	Glu	Leu	Thr	Gly	Tyr	Gly	Thr	Val	Thr	Met	Glu	Cys	Ser	
		340				345						350				
ccg	aga	aca	ggc	ctc	gac	ttc	aat	gag	atg	gtg	ttg	ctg	cag	atg	gaa	1104
Pro	Arg	Thr	Gly	Leu	Asp	Phe	Asn	Glu	Met	Val	Leu	Gln	Met	Glu		
	355					360						365				

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aat aaa gct tgg ctg gtg cat agg caa tgg ttc cta gac ctg ccg tta Asn Lys Ala Trp Leu Val His Arg Gln Trp Phe Leu Asp Leu Pro Leu 370 375 380	1152
cca tgg ctg ccc gga gcg gac aca caa ggg tca aat tgg ata caa aaa Pro Trp Leu Pro Gly Ala Asp Thr Gln Gly Ser Asn Trp Ile Gln Lys 385 390 395 400	1200
gaa aca ttg gtc act ttc aaa aat cct cat gcg aag aaa cag gat gtt Glu Thr Leu Val Thr Phe Lys Asn Pro His Ala Lys Lys Gln Asp Val 405 410 415	1248
gtt gtt tta gga tcc caa gaa ggg gcc atg cac aca gca ctc aca ggg Val Val Leu Gly Ser Gln Glu Gly Ala Met His Thr Ala Leu Thr Gly 420 425 430	1296
gcc aca gaa atc caa atg tca tca gga aac tta ctc ttc aca gga cat Ala Thr Glu Ile Gln Met Ser Ser Gly Asn Leu Leu Phe Thr Gly His 435 440 445	1344
ctc aag tgc agg ctg aga atg gac aag cta cag ctc aaa gga atg tca Leu Lys Cys Arg Leu Arg Met Asp Lys Leu Gln Leu Lys Gly Met Ser 450 455 460	1392
tac tct atg tgc aca gga aag ttt aaa gtt gtg aag gaa ata gca gaa Tyr Ser Met Cys Thr Gly Lys Phe Lys Val Val Lys Glu Ile Ala Glu 465 470 475 480	1440
aca caa cat gga aca ata gtt atc agg gtg cag tat gaa ggg gac ggc Thr Gln His Gly Thr Ile Val Ile Arg Val Gln Tyr Glu Gly Asp Gly 485 490 495	1488
tct cca tgt aaa atc cct ttt gag ata atg gat ttg gaa aaa aga cat Ser Pro Cys Lys Ile Pro Phe Glu Ile Met Asp Leu Glu Lys Arg His 500 505 510	1536
gtc tta ggt cgc ctg atc aca gtc aac cca att gtg aca gaa aaa gat Val Leu Gly Arg Leu Ile Thr Val Asn Pro Ile Val Thr Glu Lys Asp 515 520 525	1584
agc cca gtc aac ata gaa gca gaa cct cca ttc gga gac agc tac atc Ser Pro Val Asn Ile Glu Ala Glu Pro Pro Phe Gly Asp Ser Tyr Ile 530 535 540	1632
atc ata gga gta gag ccg gga caa ctg aag ctc aac tgg ttt aag aaa Ile Ile Gly Val Glu Pro Gly Gln Leu Lys Leu Asn Trp Phe Lys Lys 545 550 555 560	1680
gga agt tct atc ggc caa atg ttt gag aca aca atg agg ggg gcg aag Gly Ser Ser Ile Gly Gln Met Phe Glu Thr Thr Met Arg Gly Ala Lys 565 570 575	1728
aga atg gcc att ttg ggt gac aca gcc tgg gat ttt gga tcc ctg gga Arg Met Ala Ile Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser Leu Gly 580 585 590	1776
gga gtg ttt aca tct ata gga aaa gcc ctc cac caa gtc ttt gga gca Gly Val Phe Thr Ser Ile Gly Lys Ala Leu His Gln Val Phe Gly Ala 595 600 605	1824
atc tat gga gct gcc ttc agt ggg gtc tca tgg act atg aaa atc ctc Ile Tyr Gly Ala Ala Phe Ser Gly Val Ser Trp Thr Met Lys Ile Leu 610 615 620	1872
ata gga gtc att atc aca tgg ata gga atg aat tca cgc agc acc tca Ile Gly Val Ile Ile Thr Trp Ile Gly Met Asn Ser Arg Ser Thr Ser 625 630 635 640	1920
ctg tct gtg tca cta gta ttg gtg gga gtc gtg acg ctg tat ttg gga Leu Ser Val Ser Leu Val Leu Val Gly Val Val Thr Leu Tyr Leu Gly 645 650 655	1968
gtt atg gtg ggc gcc Val Met Val Gly Ala 660	1983



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&lt;211&gt; LENGTH: 661

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Dengue-2 virus

&lt;400&gt; SEQUENCE: 2

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Phe His Leu Thr Thr Arg Asn Gly Glu Pro His Met Ile Val Ser Arg
 1           5           10          15

Gln Glu Lys Gly Lys Ser Leu Leu Phe Lys Thr Glu Asp Gly Val Asn
 20          25          30

Met Cys Thr Leu Met Ala Met Asp Leu Gly Glu Leu Cys Glu Asp Thr
 35          40          45

Ile Thr Tyr Lys Cys Pro Leu Leu Arg Gln Asn Glu Pro Glu Asp Ile
 50          55          60

Asp Cys Trp Cys Asn Ser Thr Ser Thr Trp Val Thr Tyr Gly Thr Cys
 65          70          75          80

Thr Thr Thr Gly Glu His Arg Arg Glu Lys Arg Ser Val Ala Leu Val
 85          90          95

Pro His Val Gly Met Gly Leu Glu Thr Arg Thr Glu Thr Trp Met Ser
100          105          110

Ser Glu Gly Ala Trp Lys His Ala Gln Arg Ile Glu Ile Trp Ile Leu
115          120          125

Arg His Pro Gly Phe Thr Ile Met Ala Ala Ile Leu Ala Tyr Thr Ile
130          135          140

Gly Thr Thr His Phe Gln Arg Ala Leu Ile Phe Ile Leu Leu Thr Ala
145          150          155          160

Val Ala Pro Ser Met Thr Met Arg Cys Ile Gly Ile Ser Asn Arg Asp
165          170          175

Phe Val Glu Gly Val Ser Gly Gly Ser Trp Val Asp Ile Val Leu Glu
180          185          190

His Gly Ser Cys Val Thr Thr Met Ala Lys Asn Lys Pro Thr Leu Asp
195          200          205

Phe Glu Leu Ile Lys Thr Glu Ala Lys Gln Pro Ala Thr Leu Arg Lys
210          215          220

Tyr Cys Ile Glu Ala Lys Leu Thr Asn Thr Thr Thr Glu Ser Arg Cys
225          230          235          240

Pro Thr Gln Gly Glu Pro Ser Leu Asn Glu Glu Gln Asp Lys Arg Phe
245          250          255

Val Cys Lys His Ser Met Val Asp Arg Gly Trp Gly Asn Gly Cys Gly
260          265          270

Leu Phe Gly Lys Gly Gly Ile Val Thr Cys Ala Met Phe Thr Cys Lys
275          280          285

Lys Asn Met Glu Gly Lys Val Val Gln Pro Glu Asn Leu Glu Tyr Thr
290          295          300

Ile Val Val Thr Pro His Ser Gly Glu Glu His Ala Val Gly Asn Asp
305          310          315          320

Thr Gly Lys His Gly Lys Glu Ile Lys Val Thr Pro Gln Ser Ser Ile
325          330          335

Thr Glu Ala Glu Leu Thr Gly Tyr Gly Thr Val Thr Met Glu Cys Ser
340          345          350

Pro Arg Thr Gly Leu Asp Phe Asn Glu Met Val Leu Leu Gln Met Glu
355          360          365

Asn Lys Ala Trp Leu Val His Arg Gln Trp Phe Leu Asp Leu Pro Leu
370          375          380

Pro Trp Leu Pro Gly Ala Asp Thr Gln Gly Ser Asn Trp Ile Gln Lys

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385	390	395	400
Glu Thr Leu Val Thr Phe Lys Asn Pro His Ala Lys Lys Gln Asp Val			
	405	410	415
Val Val Leu Gly Ser Gln Glu Gly Ala Met His Thr Ala Leu Thr Gly			
	420	425	430
Ala Thr Glu Ile Gln Met Ser Ser Gly Asn Leu Leu Phe Thr Gly His			
	435	440	445
Leu Lys Cys Arg Leu Arg Met Asp Lys Leu Gln Leu Lys Gly Met Ser			
	450	455	460
Tyr Ser Met Cys Thr Gly Lys Phe Lys Val Val Lys Glu Ile Ala Glu			
	465	470	475
Thr Gln His Gly Thr Ile Val Ile Arg Val Gln Tyr Glu Gly Asp Gly			
	485	490	495
Ser Pro Cys Lys Ile Pro Phe Glu Ile Met Asp Leu Glu Lys Arg His			
	500	505	510
Val Leu Gly Arg Leu Ile Thr Val Asn Pro Ile Val Thr Glu Lys Asp			
	515	520	525
Ser Pro Val Asn Ile Glu Ala Glu Pro Pro Phe Gly Asp Ser Tyr Ile			
	530	535	540
Ile Ile Gly Val Glu Pro Gly Gln Leu Lys Leu Asn Trp Phe Lys Lys			
	545	550	555
Gly Ser Ser Ile Gly Gln Met Phe Glu Thr Thr Met Arg Gly Ala Lys			
	565	570	575
Arg Met Ala Ile Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser Leu Gly			
	580	585	590
Gly Val Phe Thr Ser Ile Gly Lys Ala Leu His Gln Val Phe Gly Ala			
	595	600	605
Ile Tyr Gly Ala Ala Phe Ser Gly Val Ser Trp Thr Met Lys Ile Leu			
	610	615	620
Ile Gly Val Ile Ile Thr Trp Ile Gly Met Asn Ser Arg Ser Thr Ser			
	625	630	635
Leu Ser Val Ser Leu Val Leu Val Gly Val Val Thr Leu Tyr Leu Gly			
	645	650	655
Val Met Val Gly Ala			
	660		

<210> SEQ ID NO 3  
 <211> LENGTH: 7  
 <212> TYPE: PRT  
 <213> ORGANISM: Japanese Encephalitis virus

<400> SEQUENCE: 3

Tyr Ala Gly Ala Met Lys Leu  
 1 5

<210> SEQ ID NO 4  
 <211> LENGTH: 7  
 <212> TYPE: PRT  
 <213> ORGANISM: Yellow Fever virus

<400> SEQUENCE: 4

Met Thr Gly Gly Val Thr Leu  
 1 5

<210> SEQ ID NO 5  
 <211> LENGTH: 7  
 <212> TYPE: PRT

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<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: derived from Japanese Encephalitis virus and  
Yellow Fever virus

<400> SEQUENCE: 5

Met Thr Gly Gly Met Lys Leu  
1 5

<210> SEQ ID NO 6  
<211> LENGTH: 7  
<212> TYPE: PRT  
<213> ORGANISM: Japanese Encephalitis virus

<400> SEQUENCE: 6

Asn Lys Arg Gly Gly Asn Glu  
1 5

<210> SEQ ID NO 7  
<211> LENGTH: 7  
<212> TYPE: PRT  
<213> ORGANISM: Yellow Fever virus

<400> SEQUENCE: 7

Lys Arg Arg Ser His Asp Val  
1 5

<210> SEQ ID NO 8  
<211> LENGTH: 7  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: derived from Yellow Fever Virus and Japanese  
Encephalitis Virus

<400> SEQUENCE: 8

Lys Arg Arg Ser His Asp Val  
1 5

<210> SEQ ID NO 9  
<211> LENGTH: 10  
<212> TYPE: PRT  
<213> ORGANISM: Japanese Encephalitis virus

<400> SEQUENCE: 9

Thr Asn Val His Ala Asp Thr Gly Cys Ala  
1 5 10

<210> SEQ ID NO 10  
<211> LENGTH: 10  
<212> TYPE: PRT  
<213> ORGANISM: Yellow Fever virus

<400> SEQUENCE: 10

Leu Gly Val Gly Ala Asp Gln Gly Cys Ala  
1 5 10

<210> SEQ ID NO 11  
<211> LENGTH: 10  
<212> TYPE: PRT  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: derived from Japanese Encephalitis virus and  
Yellow Fever virus

<400> SEQUENCE: 11

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Thr Asn Val Gly Ala Asp Gln Gly Cys Ala  
1 5 10

<210> SEQ ID NO 12

<211> LENGTH: 10892

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: derived from Japanese Encephalitis virus and Yellow Fever virus

<221> NAME/KEY: CDS

<222> LOCATION: (119)...(10381)

<400> SEQUENCE: 12

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agtaaatcct gtgtgctaat tgaggtgcat tggctctgcaa atcgagttgc taggcaataa      60
acacatttgg attaatTTta atcgttctgtt gagcgattag cagagaactg accagaac      118
atg tct ggt cgt aaa gct cag gga aaa acc ctg ggc gtc aat atg gta      166
Met Ser Gly Arg Lys Ala Gln Gly Lys Thr Leu Gly Val Asn Met Val
1 5 10 15
cga cga gga gtt cgc tcc ttg tca aac aaa ata aaa caa aaa aca aaa      214
Arg Arg Gly Val Arg Ser Leu Ser Asn Lys Ile Lys Gln Lys Thr Lys
20 25 30
caa att gga aac aga cct gga cct tca aga ggt gtt caa gga ttt atc      262
Gln Ile Gly Asn Arg Pro Gly Pro Ser Arg Gly Val Gln Gly Phe Ile
35 40 45
ttt ttc ttt ttg ttc aac att ttg act gga aaa aag atc aca gcc cac      310
Phe Phe Phe Leu Phe Asn Ile Leu Thr Gly Lys Lys Ile Thr Ala His
50 55 60
cta aag agg ttg tgg aaa atg ctg gac cca aga caa ggc ttg gct gtt      358
Leu Lys Arg Leu Trp Lys Met Leu Asp Pro Arg Gln Gly Leu Ala Val
65 70 75 80
cta agg aaa gtc aag aga gtg gtg gcc agt ttg atg aga gga ttg tcc      406
Leu Arg Lys Val Lys Arg Val Val Ala Ser Leu Met Arg Gly Leu Ser
85 90 95
tca agg aaa cgc cgt tcc cat gat gtt ctg act gtg caa ttc cta att      454
Ser Arg Lys Arg Arg Ser His Asp Val Leu Thr Val Gln Phe Leu Ile
100 105 110
ttg gga atg ctg ttg atg acg ggt gga atg aag ttg tcg aat ttc cag      502
Leu Gly Met Leu Leu Met Thr Gly Gly Met Lys Leu Ser Asn Phe Gln
115 120 125
ggg aag ctt ttg atg acc atc aac aac acg gac att gca gac gtt atc      550
Gly Lys Leu Leu Met Thr Ile Asn Asn Thr Asp Ile Ala Asp Val Ile
130 135 140
gtg att ccc acc tca aaa gga gag aac aga tgt tgg gtt cgg gca atc      598
Val Ile Pro Thr Ser Lys Gly Glu Asn Arg Cys Trp Val Arg Ala Ile
145 150 155 160
gac gtc ggc tac atg tgt gag gac act atc acg tac gaa tgt cct aag      646
Asp Val Gly Tyr Met Cys Glu Asp Thr Ile Thr Tyr Glu Cys Pro Lys
165 170 175
ctt acc atg ggc aat gat cca gag gat gtg gat tgc tgg tgt gac aac      694
Leu Thr Met Gly Asn Asp Pro Glu Asp Val Asp Cys Trp Cys Asp Asn
180 185 190
caa gaa gtc tac gtc caa tat gga cgg tgc acg cgg acc agg cat tcc      742
Gln Glu Val Tyr Val Gln Tyr Gly Arg Cys Thr Arg Thr Arg His Ser
195 200 205
aag cga agc agg aga tcc gtg tcg gtc caa aca cat ggg gag agt tca      790
Lys Arg Ser Arg Arg Ser Val Ser Val Gln Thr His Gly Glu Ser Ser
210 215 220
cta gtg aat aaa aaa gag gct tgg ctg gat tca acg aaa gcc aca cga      838
Leu Val Asn Lys Lys Glu Ala Trp Leu Asp Ser Thr Lys Ala Thr Arg
225 230 235 240

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tat ctc atg aaa act gag aac tgg atc ata agg aat cct ggc tat gct Tyr Leu Met Lys Thr Glu Asn Trp Ile Ile Arg Asn Pro Gly Tyr Ala 245 250 255	886
ttc ctg gcg gcg gta ctt ggc tgg atg ctt ggc agt aac aac ggt caa Phe Leu Ala Ala Val Leu Gly Trp Met Leu Gly Ser Asn Asn Gly Gln 260 265 270	934
cgc gtg gta ttt acc atc ctc ctg ctg ttg gtc gct ccg gct tac agt Arg Val Val Phe Thr Ile Leu Leu Leu Leu Val Ala Pro Ala Tyr Ser 275 280 285	982
ttt aat tgt ctg gga atg ggc aat cgt gac ttc ata gaa gga gcc agt Phe Asn Cys Leu Gly Met Gly Asn Arg Asp Phe Ile Glu Gly Ala Ser 290 295 300	1030
ggg gcc act tgg gtg gac ttg gtg cta gaa gga gac agc tgc ttg aca Gly Ala Thr Trp Val Asp Leu Val Leu Glu Gly Asp Ser Cys Leu Thr 305 310 315 320	1078
atc atg gca aac gac aaa cca aca ttg gac gtc cgc atg att aac atc Ile Met Ala Asn Asp Lys Pro Thr Leu Asp Val Arg Met Ile Asn Ile 325 330 335	1126
gaa gct agc caa ctt gct gag gtc aga agt tac tgc tat cat gct tca Glu Ala Ser Gln Leu Ala Glu Val Arg Ser Tyr Cys Tyr His Ala Ser 340 345 350	1174
gtc act gac atc tcg acg gtg gct cgg tgc ccc acg act gga gaa gcc Val Thr Asp Ile Ser Thr Val Ala Arg Cys Pro Thr Thr Gly Glu Ala 355 360 365	1222
cac aac gag aag cga gct gat agt agc tat gtg tgc aaa caa ggc ttc His Asn Glu Lys Arg Ala Asp Ser Ser Tyr Val Cys Lys Gln Gly Phe 370 375 380	1270
act gac cgt ggg tgg ggc aac gga tgt gga ttt ttc ggg aag gga agc Thr Asp Arg Gly Trp Gly Asn Gly Cys Gly Phe Phe Gly Lys Gly Ser 385 390 395 400	1318
att gac aca tgt gca aaa ttc tcc tgc acc agt aaa gcg att ggg aga Ile Asp Thr Cys Ala Lys Phe Ser Cys Thr Ser Lys Ala Ile Gly Arg 405 410 415	1366
aca atc cag cca gaa aac atc aaa tac aaa gtt ggc att ttt gtg cat Thr Ile Gln Pro Glu Asn Ile Lys Tyr Lys Val Gly Ile Phe Val His 420 425 430	1414
gga acc acc act tcg gaa aac cat ggg aat tat tca gcg caa gtt ggg Gly Thr Thr Thr Ser Glu Asn His Gly Asn Tyr Ser Ala Gln Val Gly 435 440 445	1462
gcg tcc cag gcg gca aag ttt aca gta aca ccc aat gct cct tcg gta Ala Ser Gln Ala Ala Lys Phe Thr Val Thr Pro Asn Ala Pro Ser Val 450 455 460	1510
gcc ctc aaa ctt ggt gac tac gga gaa gtc aca ctg gac tgt gag cca Ala Leu Lys Leu Gly Asp Tyr Gly Glu Val Thr Leu Asp Cys Glu Pro 465 470 475 480	1558
agg agt gga ctg aac act gaa gcg ttt tac gtc atg acc gtg ggg tca Arg Ser Gly Leu Asn Thr Glu Ala Phe Tyr Val Met Thr Val Gly Ser 485 490 495	1606
aag tca ttt ctg gtc cat agg gag tgg ttt cat gac ctc gct ctc ccc Lys Ser Phe Leu Val His Arg Glu Trp Phe His Asp Leu Ala Leu Pro 500 505 510	1654
tgg acg tcc cct tcg agc aca gcg tgg aga aac aga gaa ctc ctc atg Trp Thr Ser Pro Ser Ser Thr Ala Trp Arg Asn Arg Glu Leu Leu Met 515 520 525	1702
gaa ttt gaa ggg gcg cac gcc aca aaa cag tcc gtt gtt gct ctt ggg Glu Phe Glu Gly Ala His Ala Thr Lys Gln Ser Val Val Ala Leu Gly 530 535 540	1750
tca cag gaa gga ggc ctc cat cat gcg ttg gca gga gcc atc gtg gtg Ser Gln Glu Gly Gly Leu His His Ala Leu Ala Gly Ala Ile Val Val 545 550 555	1798

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545	550	555	560	
gag tac tca agc tca gtg atg tta aca tca ggc cac ctg aaa tgt agg Glu Tyr Ser Ser Val Met Leu Thr Ser Gly His Leu Lys Cys Arg 565 570 575				1846
ctg aaa atg gac aaa ctg gct ctg aaa ggc aca acc tat ggc atg tgt Leu Lys Met Asp Lys Leu Ala Leu Lys Gly Thr Thr Tyr Gly Met Cys 580 585 590				1894
aca gaa aaa ttc tcg ttc gcg aaa aat ccg gtg gac act ggt cac gga Thr Glu Lys Phe Ser Phe Ala Lys Asn Pro Val Asp Thr Gly His Gly 595 600 605				1942
aca gtt gtc att gaa ctc tcc tac tct ggg agt gat ggc ccc tgc aaa Thr Val Val Ile Glu Leu Ser Tyr Ser Gly Ser Asp Gly Pro Cys Lys 610 615 620				1990
att ccg att gtt tcc gtt gcg agc ctc aat gac atg acc ccc gtt ggg Ile Pro Ile Val Ser Val Ala Ser Leu Asn Asp Met Thr Pro Val Gly 625 630 635 640				2038
cgg ctg gtg aca gtg aac ccc ttc gtc gcg act tcc agt gcc aac tca Arg Leu Val Thr Val Asn Pro Phe Val Ala Thr Ser Ser Ala Asn Ser 645 650 655				2086
aag gtg ctg gtc gag atg gaa ccc ccc ttc gga gac tcc tac atc gta Lys Val Leu Val Glu Met Glu Pro Pro Phe Gly Asp Ser Tyr Ile Val 660 665 670				2134
gtt gga agg gga gac aag cag atc aac cac cat tgg cac aaa gct gga Val Gly Arg Gly Asp Lys Gln Ile Asn His His Trp His Lys Ala Gly 675 680 685				2182
agc acg ctg ggc aag gcc ttt tca aca act ttg aag gga gct caa aga Ser Thr Leu Gly Lys Ala Phe Ser Thr Thr Leu Lys Gly Ala Gln Arg 690 695 700				2230
ctg gca gcg ttg ggc gac aca gcc tgg gac ttt ggc tct att gga ggg Leu Ala Ala Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser Ile Gly Gly 705 710 715 720				2278
gtc ttc aac tcc ata gga aga gcc gtt cac caa gtg ttt ggt ggt gcc Val Phe Asn Ser Ile Gly Arg Ala Val His Gln Val Phe Gly Gly Ala 725 730 735				2326
ttc aga aca ctc ttt ggg gga atg tct tgg atc aca caa ggg cta atg Phe Arg Thr Leu Phe Gly Gly Met Ser Trp Ile Thr Gln Gly Leu Met 740 745 750				2374
ggt gcc cta ctg ctc tgg atg ggc gtc aac gca cga gac cga tca att Gly Ala Leu Leu Leu Trp Met Gly Val Asn Ala Arg Asp Arg Ser Ile 755 760 765				2422
gct ttg gcc ttc tta gcc aca gga ggt gtg ctc gtg ttc tta gcg acc Ala Leu Ala Phe Leu Ala Thr Gly Gly Val Leu Val Phe Leu Ala Thr 770 775 780				2470
aat gtg ggc gcc gat caa gga tgc gcc atc aac ttt ggc aag aga gag Asn Val Gly Ala Asp Gln Gly Cys Ala Ile Asn Phe Gly Lys Arg Glu 785 790 795 800				2518
ctc aag tgc gga gat ggt atc ttc ata ttt aga gac tct gat gac tgg Leu Lys Cys Gly Asp Gly Ile Phe Ile Phe Arg Asp Ser Asp Asp Trp 805 810 815				2566
ctg aac aag tac tca tac tat cca gaa gat cct gtg aag ctt gca tca Leu Asn Lys Tyr Ser Tyr Tyr Pro Glu Asp Pro Val Lys Leu Ala Ser 820 825 830				2614
ata gtg aaa gcc tct ttt gaa gaa ggg aag tgt ggc cta aat tca gtt Ile Val Lys Ala Ser Phe Glu Glu Gly Lys Cys Gly Leu Asn Ser Val 835 840 845				2662
gac tcc ctt gag cat gag atg tgg aga agc agg gca gat gag atc aat Asp Ser Leu Glu His Glu Met Trp Arg Ser Arg Ala Asp Glu Ile Asn 850 855 860				2710
gcc att ttt gag gaa aac gag gtg gac att tct gtt gtc gtg cag gat				2758

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Ala 865	Ile	Phe	Glu	Glu	Asn 870	Glu	Val	Asp	Ile	Ser 875	Val	Val	Val	Gln	Asp 880	
cca	aag	aat	gtt	tac	cag	aga	gga	act	cat	cca	ttt	tcc	aga	att	cgg	2806
Pro	Lys	Asn	Val	Tyr	Gln	Arg	Gly	Thr	His	Pro	Phe	Ser	Arg	Ile	Arg	
				885					890					895		
gat	ggt	ctg	cag	tat	ggt	tgg	aag	act	tgg	ggt	aag	aac	ctt	gtg	ttc	2854
Asp	Gly	Leu	Gln	Tyr	Gly	Trp	Lys	Thr	Trp	Gly	Lys	Asn	Leu	Val	Phe	
				900				905					910			
tcc	cca	ggg	agg	aag	aat	gga	agc	ttc	atc	ata	gat	gga	aag	tcc	agg	2902
Ser	Pro	Gly	Arg	Lys	Asn	Gly	Ser	Phe	Ile	Ile	Asp	Gly	Lys	Ser	Arg	
		915					920					925				
aaa	gaa	tgc	ccg	ttt	tca	aac	cgg	gtc	tgg	aat	tct	ttc	cag	ata	gag	2950
Lys	Glu	Cys	Pro	Phe	Ser	Asn	Arg	Val	Trp	Asn	Ser	Phe	Gln	Ile	Glu	
	930					935					940					
gag	ttt	ggg	acg	gga	gtg	ttc	acc	aca	cgc	gtg	tac	atg	gac	gca	gtc	2998
Glu	Phe	Gly	Thr	Gly	Val	Phe	Thr	Thr	Arg	Val	Tyr	Met	Asp	Ala	Val	
945				950					955						960	
ttt	gaa	tac	acc	ata	gac	tgc	gat	gga	tct	atc	ttg	ggt	gca	gcg	gtg	3046
Phe	Glu	Tyr	Thr	Ile	Asp	Cys	Asp	Gly	Ser	Ile	Leu	Gly	Ala	Ala	Val	
				965				970						975		
aac	gga	aaa	aag	agt	gcc	cat	ggc	tct	cca	aca	ttt	tgg	atg	gga	agt	3094
Asn	Gly	Lys	Lys	Ser	Ala	His	Gly	Ser	Pro	Thr	Phe	Trp	Met	Gly	Ser	
		980					985					990				
cat	gaa	gta	aat	ggg	aca	tgg	atg	atc	cac	acc	ttg	gag	gca	tta	gat	3142
His	Glu	Val	Asn	Gly	Thr	Trp	Met	Ile	His	Thr	Leu	Glu	Ala	Leu	Asp	
		995					1000					1005				
tac	aag	gag	tgt	gag	tgg	cca	ctg	aca	cat	acg	att	gga	aca	tca	gtt	3190
Tyr	Lys	Glu	Cys	Glu	Trp	Pro	Leu	Thr	His	Thr	Ile	Gly	Thr	Ser	Val	
	1010					1015					1020					
gaa	gag	agt	gaa	atg	ttc	atg	ccg	aga	tca	atc	gga	ggc	cca	gtt	agc	3238
Glu	Glu	Ser	Glu	Met	Phe	Met	Pro	Arg	Ser	Ile	Gly	Gly	Pro	Val	Ser	
1025				1030					1035					1040		
tct	cac	aat	cat	atc	cct	gga	tac	aag	gtt	cag	acg	aac	gga	cct	tgg	3286
Ser	His	Asn	His	Ile	Pro	Gly	Tyr	Lys	Val	Gln	Thr	Asn	Gly	Pro	Trp	
				1045				1050					1055			
atg	cag	gta	cca	cta	gaa	gtg	aag	aga	gaa	gct	tgc	cca	ggg	act	agc	3334
Met	Gln	Val	Pro	Leu	Glu	Val	Lys	Arg	Glu	Ala	Cys	Pro	Gly	Thr	Ser	
		1060						1065					1070			
gtg	atc	att	gat	ggc	aac	tgt	gat	gga	cgg	gga	aaa	tca	acc	aga	tcc	3382
Val	Ile	Ile	Asp	Gly	Asn	Cys	Asp	Gly	Arg	Gly	Lys	Ser	Thr	Arg	Ser	
	1075					1080						1085				
acc	acg	gat	agc	ggg	aaa	gtt	att	cct	gaa	tgg	tgt	tgc	cgc	tcc	tgc	3430
Thr	Thr	Asp	Ser	Gly	Lys	Val	Ile	Pro	Glu	Trp	Cys	Cys	Arg	Ser	Cys	
	1090					1095					1100					
aca	atg	ccg	cct	gtg	agc	ttc	cat	ggt	agt	gat	ggg	tgt	tgg	tat	ccc	3478
Thr	Met	Pro	Pro	Val	Ser	Phe	His	Gly	Ser	Asp	Gly	Cys	Trp	Tyr	Pro	
	1105				1110					1115				1120		
atg	gaa	att	agg	cca	agg	aaa	acg	cat	gaa	agc	cat	ctg	gtg	cgc	tcc	3526
Met	Glu	Ile	Arg	Pro	Arg	Lys	Thr	His	Glu	Ser	His	Leu	Val	Arg	Ser	
				1125				1130					1135			
tgg	gtt	aca	gct	gga	gaa	ata	cat	gct	gtc	cct	ttt	ggt	ttg	gtg	agc	3574
Trp	Val	Thr	Ala	Gly	Glu	Ile	His	Ala	Val	Pro	Phe	Gly	Leu	Val	Ser	
		1140						1145					1150			
atg	atg	ata	gca	atg	gaa	gtg	gtc	cta	agg	aaa	aga	cag	gga	cca	aag	3622
Met	Met	Ile	Ala	Met	Glu	Val	Val	Leu	Arg	Lys	Arg	Gln	Gly	Pro	Lys	
		1155					1160					1165				
caa	atg	ttg	gtt	gga	gga	gta	gtg	ctc	ttg	gga	gca	atg	ctg	gtc	ggg	3670
Gln	Met	Leu	Val	Gly	Gly	Val	Val	Leu	Leu	Gly	Ala	Met	Leu	Val	Gly	
	1170					1175					1180					

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caa gta act ctc ctt gat ttg ctg aaa ctc aca gtg gct gtg gga ttg	3718
Gln Val Thr Leu Leu Asp Leu Leu Lys Leu Thr Val Ala Val Gly Leu	
1185 1190 1195 1200	
cat ttc cat gag atg aac aat gga gga gac gcc atg tat atg gcg ttg	3766
His Phe His Glu Met Asn Asn Gly Gly Asp Ala Met Tyr Met Ala Leu	
1205 1210 1215	
att gct gcc ttt tca atc aga cca ggg ctg ctc atc ggc ttt ggg ctc	3814
Ile Ala Ala Phe Ser Ile Arg Pro Gly Leu Leu Ile Gly Phe Gly Leu	
1220 1225 1230	
agg acc cta tgg agc cct cgg gaa cgc ctt gtg ctg acc cta gga gca	3862
Arg Thr Leu Trp Ser Pro Arg Glu Arg Leu Val Leu Thr Leu Gly Ala	
1235 1240 1245	
gcc atg gtg gag att gcc ttg ggt ggc gtg atg ggc ggc ctg tgg aag	3910
Ala Met Val Glu Ile Ala Leu Gly Gly Val Met Gly Gly Leu Trp Lys	
1250 1255 1260	
tat cta aat gca gtt tct ctc tgc atc ctg aca ata aat gct gtt gct	3958
Tyr Leu Asn Ala Val Ser Leu Cys Ile Leu Thr Ile Asn Ala Val Ala	
1265 1270 1275 1280	
tct agg aaa gca tca aat acc atc ttg ccc ctc atg gct ctg ttg aca	4006
Ser Arg Lys Ala Ser Asn Thr Ile Leu Pro Leu Met Ala Leu Leu Thr	
1285 1290 1295	
cct gtc act atg gct gag gtg aga ctt gcc gca atg ttc ttt tgt gcc	4054
Pro Val Thr Met Ala Glu Val Arg Leu Ala Ala Met Phe Phe Cys Ala	
1300 1305 1310	
atg gtt atc ata ggg gtc ctt cac cag aat ttc aag gac acc tcc atg	4102
Met Val Ile Ile Gly Val Leu His Gln Phe Lys Asp Thr Ser Met	
1315 1320 1325	
cag aag act ata cct ctg gtg gcc ctc aca ctc aca tct tac ctg ggc	4150
Gln Lys Thr Ile Pro Leu Val Ala Leu Thr Leu Thr Ser Tyr Leu Gly	
1330 1335 1340	
ttg aca caa cct ttt ttg ggc ctg tgt gca ttt ctg gca acc cgc ata	4198
Leu Thr Gln Pro Phe Leu Gly Leu Cys Ala Phe Leu Ala Thr Arg Ile	
1345 1350 1355 1360	
ttt ggg cga agg agt atc cca gtg aat gag gca ctc gca gca gct ggt	4246
Phe Gly Arg Arg Ser Ile Pro Val Asn Glu Ala Leu Ala Ala Ala Gly	
1365 1370 1375	
cta gtg gga gtg ctg gca gga ctg gct ttt cag gag atg gag aac ttc	4294
Leu Val Gly Val Leu Ala Gly Leu Ala Phe Gln Glu Met Glu Asn Phe	
1380 1385 1390	
ctt ggt ccg att gca gtt gga gga ctc ctg atg atg ctg gtt agc gtg	4342
Leu Gly Pro Ile Ala Val Gly Gly Leu Leu Met Met Leu Val Ser Val	
1395 1400 1405	
gct ggg agg gtg gat ggg cta gag ctc aag aag ctt ggt gaa gtt tca	4390
Ala Gly Arg Val Asp Gly Leu Glu Leu Lys Lys Leu Gly Glu Val Ser	
1410 1415 1420	
tgg gaa gag gag gcg gag atc agc ggg agt tcc gcc cgc tat gat gtg	4438
Trp Glu Glu Glu Ala Glu Ile Ser Gly Ser Ser Ala Arg Tyr Asp Val	
1425 1430 1435 1440	
gca ctc agt gaa caa ggg gag ttc aag ctg ctt tct gaa gag aaa gtg	4486
Ala Leu Ser Glu Gln Gly Glu Phe Lys Leu Leu Ser Glu Glu Lys Val	
1445 1450 1455	
cca tgg gac cag gtt gtg atg acc tcg ctg gcc ttg gtt ggg gct gcc	4534
Pro Trp Asp Gln Val Val Met Thr Ser Leu Ala Leu Val Gly Ala Ala	
1460 1465 1470	
ctc cat cca ttt gct ctt ctg ctg gtc ctt gct ggg tgg ctg ttt cat	4582
Leu His Pro Phe Ala Leu Leu Leu Val Leu Ala Gly Trp Leu Phe His	
1475 1480 1485	
gtc agg gga gct agg aga agt ggg gat gtc ttg tgg gat att ccc act	4630
Val Arg Gly Ala Arg Arg Ser Gly Asp Val Leu Trp Asp Ile Pro Thr	
1490 1495 1500	



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cct aag atc atc gag gaa tgt gaa cat ctg gag gat ggg att tat ggc Pro Lys Ile Ile Glu Glu Cys Glu His Leu Glu Asp Gly Ile Tyr Gly 1505 1510 1515 1520	4678
ata ttc cag tca acc ttc ttg ggg gcc tcc cag cga gga gtg gga gtg Ile Phe Gln Ser Thr Phe Leu Gly Ala Ser Gln Arg Gly Val Gly Val 1525 1530 1535	4726
gca cag gga ggg gtg ttc cac aca atg tgg cat gtc aca aga gga gct Ala Gln Gly Gly Val Phe His Thr Met Trp His Val Thr Arg Gly Ala 1540 1545 1550	4774
ttc ctt gtc agg aat ggc aag aag ttg att cca tct tgg gct tca gta Phe Leu Val Arg Asn Gly Lys Lys Leu Ile Pro Ser Trp Ala Ser Val 1555 1560 1565	4822
aag gaa gac ctt gtc gcc tat ggt ggc tca tgg aag ttg gaa ggc aga Lys Glu Asp Leu Val Ala Tyr Gly Gly Ser Trp Lys Leu Glu Gly Arg 1570 1575 1580	4870
tgg gat gga gag gaa gag gtc cag ttg atc gcg gct gtt cca gga aag Trp Asp Gly Glu Glu Val Gln Leu Ile Ala Ala Val Pro Gly Lys 1585 1590 1595 1600	4918
aac gtg gtc aac gtc cag aca aaa ccg agc ttg ttc aaa gtg agg aat Asn Val Val Asn Val Gln Thr Lys Pro Ser Leu Phe Lys Val Arg Asn 1605 1610 1615	4966
ggg gga gaa atc ggg gct gtc gct ctt gac tat ccg agt ggc act tca Gly Gly Glu Ile Gly Ala Val Ala Leu Asp Tyr Pro Ser Gly Thr Ser 1620 1625 1630	5014
gga tct cct att gtt aac agg aac gga gag gtg att ggg ctg tac ggc Gly Ser Pro Ile Val Asn Arg Asn Gly Glu Val Ile Gly Leu Tyr Gly 1635 1640 1645	5062
aat ggc atc ctt gtc ggt gac aac tcc ttc gtg tcc gcc ata tcc cag Asn Gly Ile Leu Val Gly Asp Asn Ser Phe Val Ser Ala Ile Ser Gln 1650 1655 1660	5110
act gag gtg aag gaa gaa gga aag gag gag ctc caa gag atc ccg aca Thr Glu Val Lys Glu Glu Gly Lys Glu Glu Leu Gln Glu Ile Pro Thr 1665 1670 1675 1680	5158
atg cta aag aaa gga atg aca act gtc ctt gat ttt cat cct gga gct Met Leu Lys Lys Gly Met Thr Thr Val Leu Asp Phe His Pro Gly Ala 1685 1690 1695	5206
ggg aag aca aga cgt ttc ctc cca cag atc ttg gcc gag tgc gca cgg Gly Lys Thr Arg Arg Phe Leu Pro Gln Ile Leu Ala Glu Cys Ala Arg 1700 1705 1710	5254
aga cgc ttg cgc act ctt gtg ttg gcc ccc acc agg gtt gtt ctt tct Arg Arg Leu Arg Thr Leu Val Leu Ala Pro Thr Arg Val Val Leu Ser 1715 1720 1725	5302
gaa atg aag gag gct ttt cac ggc ctg gac gtg aaa ttc cac aca cag Glu Met Lys Glu Ala Phe His Gly Leu Asp Val Lys Phe His Thr Gln 1730 1735 1740	5350
gct ttt tcc gct cac ggc agc ggg aga gaa gtc att gat gcc atg tgc Ala Phe Ser Ala His Gly Ser Gly Arg Glu Val Ile Asp Ala Met Cys 1745 1750 1755 1760	5398
cat gcc acc cta act tac agg atg ttg gaa cca act agg gtt gtt aac His Ala Thr Leu Thr Tyr Arg Met Leu Glu Pro Thr Arg Val Val Asn 1765 1770 1775	5446
tgg gaa gtg atc att atg gat gaa gcc cat ttt ttg gat cca gct agc Trp Glu Val Ile Ile Met Asp Glu Ala His Phe Leu Asp Pro Ala Ser 1780 1785 1790	5494
ata gcc gct aga ggt tgg gca gcg cac aga gct agg gca aat gaa agt Ile Ala Ala Arg Gly Trp Ala Ala His Arg Ala Arg Ala Asn Glu Ser 1795 1800 1805	5542
gca aca atc ttg atg aca gcc aca ccg cct ggg act agt gat gaa ttt Ala Thr Ile Leu Met Thr Ala Thr Pro Pro Gly Thr Ser Asp Glu Phe 1810 1815 1820	5590

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1810	1815	1820	
cca cat tca aat ggt gaa ata gaa gat gtt caa acg gac ata ccc agt			5638
Pro His Ser Asn Gly Glu Ile Glu Asp Val Gln Thr Asp Ile Pro Ser			
1825	1830	1835	1840
gag ccc tgg aac aca ggg cat gac tgg atc ctg gct gac aaa agg ccc			5686
Glu Pro Trp Asn Thr Gly His Asp Trp Ile Leu Ala Asp Lys Arg Pro			
	1845	1850	1855
acg gca tgg ttc ctt cca tcc atc aga gct gca aat gtc atg gct gcc			5734
Thr Ala Trp Phe Leu Pro Ser Ile Arg Ala Ala Asn Val Met Ala Ala			
	1860	1865	1870
tct ttg cgt aag gct gga aag agt gtg gtg gtc ctg aac agg aaa acc			5782
Ser Leu Arg Lys Ala Gly Lys Ser Val Val Val Leu Asn Arg Lys Thr			
	1875	1880	1885
ttt gag aga gaa tac ccc acg ata aag cag aag aaa cct gac ttt ata			5830
Phe Glu Arg Glu Tyr Pro Thr Ile Lys Gln Lys Lys Pro Asp Phe Ile			
	1890	1895	1900
ttg gcc act gac ata gct gaa atg gga gcc aac ctt tgc gtg gag cga			5878
Leu Ala Thr Asp Ile Ala Glu Met Gly Ala Asn Leu Cys Val Glu Arg			
	1905	1910	1915
gtg ctg gat tgc agg acg gct ttt aag cct gtg ctt gtg gat gaa ggg			5926
Val Leu Asp Cys Arg Thr Ala Phe Lys Pro Val Leu Val Asp Glu Gly			
	1925	1930	1935
agg aag gtg gca ata aaa ggg cca ctt cgt atc tcc gca tcc tct gct			5974
Arg Lys Val Ala Ile Lys Gly Pro Leu Arg Ile Ser Ala Ser Ser Ala			
	1940	1945	1950
gct caa agg agg ggg cgc att ggg aga aat ccc aac aga gat gga gac			6022
Ala Gln Arg Arg Gly Arg Ile Gly Arg Asn Pro Asn Arg Asp Gly Asp			
	1955	1960	1965
tca tac tac tat tct gag cct aca agt gaa aat aat gcc cac cac gtc			6070
Ser Tyr Tyr Tyr Ser Glu Pro Thr Ser Glu Asn Asn Ala His His Val			
	1970	1975	1980
tgc tgg ttg gag gcc tca atg ctc ttg gac aac atg gag gtg agg ggt			6118
Cys Trp Leu Glu Ala Ser Met Leu Leu Asp Asn Met Glu Val Arg Gly			
	1985	1990	2000
gga atg gtc gcc cca ctc tat ggc gtt gaa gga act aaa aca cca gtt			6166
Gly Met Val Ala Pro Leu Tyr Gly Val Glu Gly Thr Lys Thr Pro Val			
	2005	2010	2015
tcc cct ggt gaa atg aga ctg agg gat gac cag agg aaa gtc ttc aga			6214
Ser Pro Gly Glu Met Arg Leu Arg Asp Asp Gln Arg Lys Val Phe Arg			
	2020	2025	2030
gaa cta gtg agg aat tgt gac ctg ccc gtt tgg ctt tcg tgg caa gtg			6262
Glu Leu Val Arg Asn Cys Asp Leu Pro Val Trp Leu Ser Trp Gln Val			
	2035	2040	2045
gcc aag gct ggt ttg aag acg aat gat cgt aag tgg tgt ttt gaa ggc			6310
Ala Lys Ala Gly Leu Lys Thr Asn Asp Arg Lys Trp Cys Phe Glu Gly			
	2050	2055	2060
cct gag gaa cat gag atc ttg aat gac agc ggt gaa aca gtg aag tgc			6358
Pro Glu Glu His Glu Ile Leu Asn Asp Ser Gly Glu Thr Val Lys Cys			
	2065	2070	2075
agg gct cct gga gga gca aag aag cct ctg cgc cca agg tgg tgt gat			6406
Arg Ala Pro Gly Gly Ala Lys Lys Pro Leu Arg Pro Arg Trp Cys Asp			
	2085	2090	2095
gaa agg gtg tca tct gac cag agt gcg ctg tct gaa ttt att aag ttt			6454
Glu Arg Val Ser Ser Asp Gln Ser Ala Leu Ser Glu Phe Ile Lys Phe			
	2100	2105	2110
gct gaa ggt agg agg gga gct gct gaa gtg cta gtt gtg ctg agt gaa			6502
Ala Glu Gly Arg Arg Gly Ala Ala Glu Val Leu Val Val Leu Ser Glu			
	2115	2120	2125
ctc cct gat ttc ctg gct aaa aaa ggt gga gag gca atg gat acc atc			6550

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Leu	Pro	Asp	Phe	Leu	Ala	Lys	Lys	Gly	Gly	Glu	Ala	Met	Asp	Thr	Ile	
2130						2135					2140					
agt	gtg	ttc	ctc	cac	tct	gag	gaa	ggc	tct	agg	gct	tac	cgc	aat	gca	6598
Ser	Val	Phe	Leu	His	Ser	Glu	Glu	Gly	Ser	Arg	Ala	Tyr	Arg	Asn	Ala	
2145					2150					2155					2160	
cta	tca	atg	atg	cct	gag	gca	atg	aca	ata	gtc	atg	ctg	ttt	ata	ctg	6646
Leu	Ser	Met	Met	Pro	Glu	Ala	Met	Thr	Ile	Val	Met	Leu	Phe	Ile	Leu	
				2165						2170					2175	
gct	gga	cta	ctg	aca	tcg	gga	atg	gtc	atc	ttt	ttc	atg	tct	ccc	aaa	6694
Ala	Gly	Leu	Leu	Thr	Ser	Gly	Met	Val	Ile	Phe	Phe	Met	Ser	Pro	Lys	
				2180						2185					2190	
ggc	atc	agt	aga	atg	tct	atg	gcg	atg	ggc	aca	atg	gcc	ggc	tgt	gga	6742
Gly	Ile	Ser	Arg	Met	Ser	Met	Ala	Met	Gly	Thr	Met	Ala	Gly	Cys	Gly	
		2195						2200							2205	
tat	ctc	atg	ttc	ctt	gga	ggc	gtc	aaa	ccc	act	cac	atc	tcc	tat	gtc	6790
Tyr	Leu	Met	Phe	Leu	Gly	Gly	Val	Lys	Pro	Thr	His	Ile	Ser	Tyr	Val	
		2210				2215									2220	
atg	ctc	ata	ttc	ttt	gtc	ctg	atg	gtg	gtt	gtg	atc	ccc	gag	cca	ggg	6838
Met	Leu	Ile	Phe	Phe	Val	Leu	Met	Val	Val	Ile	Pro	Glu	Pro	Gly		
		2225				2230					2235				2240	
caa	caa	agg	tcc	atc	caa	gac	aac	caa	gtg	gca	tac	ctc	att	att	ggc	6886
Gln	Gln	Arg	Ser	Ile	Gln	Asp	Asn	Gln	Val	Ala	Tyr	Leu	Ile	Ile	Gly	
				2245						2250					2255	
atc	ctg	acg	ctg	gtt	tca	gcg	gtg	gca	gcc	aac	gag	cta	ggc	atg	ctg	6934
Ile	Leu	Thr	Leu	Val	Ser	Ala	Val	Ala	Ala	Asn	Glu	Leu	Gly	Met	Leu	
				2260						2265					2270	
gag	aaa	acc	aaa	gag	gac	ctc	ttt	ggg	aag	aag	aac	tta	att	cca	tct	6982
Glu	Lys	Thr	Lys	Glu	Asp	Leu	Phe	Gly	Lys	Lys	Asn	Leu	Ile	Pro	Ser	
		2275						2280							2285	
agt	gct	tca	ccc	tgg	agt	tgg	ccg	gat	ctt	gac	ctg	aag	cca	gga	gct	7030
Ser	Ala	Ser	Pro	Trp	Ser	Trp	Pro	Asp	Leu	Asp	Leu	Lys	Pro	Gly	Ala	
		2290				2295									2300	
gcc	tgg	aca	gtg	tac	gtt	ggc	att	gtt	aca	atg	ctc	tct	cca	atg	ttg	7078
Ala	Trp	Thr	Val	Tyr	Val	Gly	Ile	Val	Thr	Met	Leu	Ser	Pro	Met	Leu	
				2305		2310					2315				2320	
cac	cac	tgg	atc	aaa	gtc	gaa	tat	ggc	aac	ctg	tct	ctg	tct	gga	ata	7126
His	His	Trp	Ile	Lys	Val	Glu	Tyr	Gly	Asn	Leu	Ser	Leu	Ser	Gly	Ile	
				2325						2330					2335	
gcc	cag	tca	gcc	tca	gtc	ctt	tct	ttc	atg	gac	aag	ggg	ata	cca	ttc	7174
Ala	Gln	Ser	Ala	Ser	Val	Leu	Ser	Phe	Met	Asp	Lys	Gly	Ile	Pro	Phe	
				2340						2345					2350	
atg	aag	atg	aat	atc	tcg	gtc	ata	atg	ctg	ctg	gtc	agt	ggc	tgg	aat	7222
Met	Lys	Met	Asn	Ile	Ser	Val	Ile	Met	Leu	Leu	Val	Ser	Gly	Trp	Asn	
				2355						2360					2365	
tca	ata	aca	gtg	atg	cct	ctg	ctc	tgt	ggc	ata	ggg	tgc	gcc	atg	ctc	7270
Ser	Ile	Thr	Val	Met	Pro	Leu	Leu	Cys	Gly	Ile	Gly	Cys	Ala	Met	Leu	
				2370						2375					2380	
cac	tgg	tct	ctc	att	tta	cct	gga	atc	aaa	gcg	cag	cag	tca	aag	ctt	7318
His	Trp	Ser	Leu	Ile	Leu	Pro	Gly	Ile	Lys	Ala	Gln	Gln	Ser	Lys	Leu	
				2385						2390					2400	
gca	cag	aga	agg	gtg	ttc	cat	ggc	gtt	gcc	aag	aac	cct	gtg	gtt	gat	7366
Ala	Gln	Arg	Arg	Val	Phe	His	Gly	Val	Ala	Lys	Asn	Pro	Val	Val	Asp	
				2405						2410					2415	
ggg	aat	cca	aca	gtt	gac	att	gag	gaa	gct	cct	gaa	atg	cct	gcc	ctt	7414
Gly	Asn	Pro	Thr	Val	Asp	Ile	Glu	Glu	Ala	Pro	Glu	Met	Pro	Ala	Leu	
				2420						2425					2430	
tat	gag	aag	aaa	ctg	gct	cta	tat	ctc	ctt	ctt	gct	ctc	agc	cta	gct	7462
Tyr	Glu	Lys	Lys	Leu	Ala	Leu	Tyr	Leu	Leu	Leu	Ala	Leu	Ser	Leu	Ala	
				2435						2440					2445	

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tct gtt gcc atg tgc aga acg ccc ttt tca ttg gct gaa ggc att gtc Ser Val Ala Met Cys Arg Thr Pro Phe Ser Leu Ala Glu Gly Ile Val 2450 2455 2460	7510
cta gca tca gct gcc tta ggg ccg ctc ata gag gga aac acc agc ctt Leu Ala Ser Ala Ala Leu Gly Pro Leu Ile Glu Gly Asn Thr Ser Leu 2465 2470 2475 2480	7558
ctt tgg aat gga ccc atg gct gtc tcc atg aca gga gtc atg agg ggg Leu Trp Asn Gly Pro Met Ala Val Ser Met Thr Gly Val Met Arg Gly 2485 2490 2495	7606
aat cac tat gct ttt gtg gga gtc atg tac aat cta tgg aag atg aaa Asn His Tyr Ala Phe Val Gly Val Met Tyr Asn Leu Trp Lys Met Lys 2500 2505 2510	7654
act gga cgc cgg ggg agc gcg aat gga aaa act ttg ggt gaa gtc tgg Thr Gly Arg Arg Gly Ser Ala Asn Gly Lys Thr Leu Gly Glu Val Trp 2515 2520 2525	7702
aag agg gaa ctg aat ctg ttg gac aag cga cag ttt gag ttg tat aaa Lys Arg Glu Leu Asn Leu Leu Asp Lys Arg Gln Phe Glu Leu Tyr Lys 2530 2535 2540	7750
agg acc gac att gtg gag gtg gat cgt gat acg gca cgc agg cat ttg Arg Thr Asp Ile Val Glu Val Asp Arg Asp Thr Ala Arg Arg His Leu 2545 2550 2555 2560	7798
gcc gaa ggg aag gtg gac acc ggg gtg gcg gtc tcc agg ggg acc gca Ala Glu Gly Lys Val Asp Thr Gly Val Ala Val Ser Arg Gly Thr Ala 2565 2570 2575	7846
aag tta agg tgg ttc cat gag cgt ggc tat gtc aag ctg gaa ggt agg Lys Leu Arg Trp Phe His Glu Arg Gly Tyr Val Lys Leu Glu Gly Arg 2580 2585 2590	7894
gtg att gac ctg ggg tgt ggc cgc gga ggc tgg tgt tac tac gct gct Val Ile Asp Leu Gly Cys Gly Arg Gly Gly Trp Cys Tyr Tyr Ala Ala 2595 2600 2605	7942
gcg caa aag gaa gtg agt ggg gtc aaa gga ttt act ctt gga aga gac Ala Gln Lys Glu Val Ser Gly Val Lys Gly Phe Thr Leu Gly Arg Asp 2610 2615 2620	7990
ggc cat gag aaa ccc atg aat gtg caa agt ctg gga tgg aac atc atc Gly His Glu Lys Pro Met Asn Val Gln Ser Leu Gly Trp Asn Ile Ile 2625 2630 2635 2640	8038
acc ttc aag gac aaa act gat atc cac cgc cta gaa cca gtg aaa tgt Thr Phe Lys Asp Lys Thr Asp Ile His Arg Leu Glu Pro Val Lys Cys 2645 2650 2655	8086
gac acc ctt ttg tgt gac att gga gag tca tca tcg tca tcg gtc aca Asp Thr Leu Leu Cys Asp Ile Gly Glu Ser Ser Ser Ser Val Thr 2660 2665 2670	8134
gag ggg gaa agg acc gtg aga gtt ctt gat act gta gaa aaa tgg ctg Glu Gly Glu Arg Thr Val Arg Val Leu Asp Thr Val Glu Lys Trp Leu 2675 2680 2685	8182
gct tgt ggg gtt gac aac ttc tgt gtg aag gtg tta gct cca tac atg Ala Cys Gly Val Asp Asn Phe Cys Val Lys Val Leu Ala Pro Tyr Met 2690 2695 2700	8230
cca gat gtt ctt gag aaa ctg gaa ttg ctc caa agg agg ttt ggc gga Pro Asp Val Leu Glu Lys Leu Glu Leu Leu Gln Arg Arg Phe Gly Gly 2705 2710 2715 2720	8278
aca gtg atc agg aac cct ctc tcc agg aat tcc act cat gaa atg tac Thr Val Ile Arg Asn Pro Leu Ser Arg Asn Ser Thr His Glu Met Tyr 2725 2730 2735	8326
tac gtg tct gga gcc cgc agc aat gtc aca ttt act gtg aac caa aca Tyr Val Ser Gly Ala Arg Ser Asn Val Thr Phe Thr Val Asn Gln Thr 2740 2745 2750	8374
tcc cgc ctc ctg atg agg aga atg agg cgt cca act gga aaa gtg acc Ser Arg Leu Leu Met Arg Arg Met Arg Arg Pro Thr Gly Lys Val Thr 2755 2760 2765	8422

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ctg gag gct gac gtc atc ctc cca att ggg aca cgc agt gtt gag aca Leu Glu Ala Asp Val Ile Leu Pro Ile Gly Thr Arg Ser Val Glu Thr 2770 2775 2780	8470
gac aag gga ccc ctg gac aaa gag gcc ata gaa gaa agg gtt gag agg Asp Lys Gly Pro Leu Asp Lys Glu Ala Ile Glu Glu Arg Val Glu Arg 2785 2790 2795 2800	8518
ata aaa tct gag tac atg acc tct tgg ttt tat gac aat gac aac ccc Ile Lys Ser Glu Tyr Met Thr Ser Trp Phe Tyr Asp Asn Asp Asn Pro 2805 2810 2815	8566
tac agg acc tgg cac tac tgt ggc tcc tat gtc aca aaa acc tcc gga Tyr Arg Thr Trp His Tyr Cys Gly Ser Tyr Val Thr Lys Thr Ser Gly 2820 2825 2830	8614
agt gcg gcg agc atg gta aat ggt gtt att aaa att ctg aca tat cca Ser Ala Ala Ser Met Val Asn Gly Val Ile Lys Ile Leu Thr Tyr Pro 2835 2840 2845	8662
tgg gac agg ata gag gag gtc aca aga atg gca atg act gac aca acc Trp Asp Arg Ile Glu Glu Val Thr Arg Met Ala Met Thr Asp Thr Thr 2850 2855 2860	8710
cct ttt gga cag caa aga gtg ttt aaa gaa aaa gtt gac acc aga gca Pro Phe Gly Gln Gln Arg Val Phe Lys Glu Lys Val Asp Thr Arg Ala 2865 2870 2875 2880	8758
aag gat cca cca gcg gga act agg aag atc atg aaa gtt gtc aac agg Lys Asp Pro Pro Ala Gly Thr Arg Lys Ile Met Lys Val Val Asn Arg 2885 2890 2895	8806
tgg ctg ttc cgc cac ctg gcc aga gaa aag aac ccc aga ctg tgc aca Trp Leu Phe Arg His Leu Ala Arg Glu Lys Asn Pro Arg Leu Cys Thr 2900 2905 2910	8854
aag gaa gaa ttt att gca aaa gtc cga agt cat gca gcc att gga gct Lys Glu Glu Phe Ile Ala Lys Val Arg Ser His Ala Ile Gly Ala 2915 2920 2925	8902
tac ctg gaa gaa caa gaa cag tgg aag act gcc aat gag gct gtc caa Tyr Leu Glu Glu Gln Glu Gln Trp Lys Thr Ala Asn Glu Ala Val Gln 2930 2935 2940	8950
gac cca aag ttc tgg gaa ctg gtg gat gaa gaa agg aag ctg cac caa Asp Pro Lys Phe Trp Glu Leu Val Asp Glu Glu Arg Lys Leu His Gln 2945 2950 2955 2960	8998
caa ggc agg tgt cgg act tgt gtg tac aac atg atg ggg aaa aga gag Gln Gly Arg Cys Arg Thr Cys Val Tyr Asn Met Met Gly Lys Arg Glu 2965 2970 2975	9046
aag aag ctg tca gag ttt ggg aaa gca aag gga agc cgt gcc ata tgg Lys Lys Leu Ser Glu Phe Gly Lys Ala Lys Gly Ser Arg Ala Ile Trp 2980 2985 2990	9094
tat atg tgg ctg gga gcg cgg tat ctt gag ttt gag gcc ctg gga ttc Tyr Met Trp Leu Gly Ala Arg Tyr Leu Glu Phe Glu Ala Leu Gly Phe 2995 3000 3005	9142
ctg aat gag gac cat tgg gct tcc agg gaa aac tca gga gga gga gtg Leu Asn Glu Asp His Trp Ala Ser Arg Glu Asn Ser Gly Gly Val 3010 3015 3020	9190
gaa ggc att ggc tta caa tac cta gga tat gtg atc aga gac ctg gct Glu Gly Ile Gly Leu Gln Tyr Leu Gly Tyr Val Ile Arg Asp Leu Ala 3025 3030 3035 3040	9238
gca atg gat ggt ggt gga ttc tac gcg gat gac acc gct gga tgg gac Ala Met Asp Gly Gly Gly Phe Tyr Ala Asp Asp Thr Ala Gly Trp Asp 3045 3050 3055	9286
acg cgc atc aca gag gca gac ctt gat gat gaa cag gag atc ttg aac Thr Arg Ile Thr Glu Ala Asp Leu Asp Asp Glu Gln Glu Ile Leu Asn 3060 3065 3070	9334
tac atg agc cca cat cac aaa aaa ctg gca caa gca gtg atg gaa atg Tyr Met Ser Pro His His Lys Lys Leu Ala Gln Ala Val Met Glu Met	9382

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3075	3080	3085	
aca tac aag aac aaa gtg gtg aaa gtg ttg aga cca gcc cca gga ggg Thr Tyr Lys Asn Lys Val Val Lys Val Leu Arg Pro Ala Pro Gly Gly 3090 3095 3100			9430
aaa gcc tac atg gat gtc ata agt cga cga gac cag aga gga tcc ggg Lys Ala Tyr Met Asp Val Ile Ser Arg Arg Asp Gln Arg Gly Ser Gly 3105 3110 3115 3120			9478
cag gta gtg act tat gct ctg aac acc atc acc aac ttg aaa gtc caa Gln Val Val Thr Tyr Ala Leu Asn Thr Ile Thr Asn Leu Lys Val Gln 3125 3130 3135			9526
ttg atc aga atg gca gaa gca gag atg gtg ata cat cac caa cat gtt Leu Ile Arg Met Ala Glu Ala Glu Met Val Ile His His Gln His Val 3140 3145 3150			9574
caa gat tgt gat gaa tca gtt ctg acc agg ctg gag gca tgg ctc act Gln Asp Cys Asp Glu Ser Val Leu Thr Arg Leu Glu Ala Trp Leu Thr 3155 3160 3165			9622
gag cac gga tgt gac aga ctg aag agg atg gcg gtg agt gga gac gac Glu His Gly Cys Asp Arg Leu Lys Arg Met Ala Val Ser Gly Asp Asp 3170 3175 3180			9670
tgt gtg gtc cgg ccc atc gat gac agg ttc ggc ctg gcc ctg tcc cat Cys Val Val Arg Pro Ile Asp Asp Arg Phe Gly Leu Ala Leu Ser His 3185 3190 3195 3200			9718
ctc aac gcc atg tcc aag gtt aga aag gac ata tct gaa tgg cag cca Leu Asn Ala Met Ser Lys Val Arg Lys Asp Ile Ser Glu Trp Gln Pro 3205 3210 3215			9766
tca aaa ggg tgg aat gat tgg gag aat gtg ccc ttc tgt tcc cac cac Ser Lys Gly Trp Asn Asp Trp Glu Asn Val Pro Phe Cys Ser His His 3220 3225 3230			9814
ttc cat gaa cta cag ctg aag gat ggc agg agg att gtg gtg cct tgc Phe His Glu Leu Gln Leu Lys Asp Gly Arg Arg Ile Val Val Pro Cys 3235 3240 3245			9862
cga gaa cag gac gag ctc att ggg aga gga agg gtg tct cca gga aac Arg Glu Gln Asp Glu Leu Ile Gly Arg Gly Arg Val Ser Pro Gly Asn 3250 3255 3260			9910
ggc tgg atg atc aag gaa aca gct tgc ctc agc aaa gcc tat gcc aac Gly Trp Met Ile Lys Glu Thr Ala Cys Leu Ser Lys Ala Tyr Ala Asn 3265 3270 3275 3280			9958
atg tgg tca ctg atg tat ttt cac aaa agg gac atg agg cta ctg tca Met Trp Ser Leu Met Tyr Phe His Lys Arg Asp Met Arg Leu Leu Ser 3285 3290 3295			10006
ttg gct gtt tcc tca gct gtt ccc acc tca tgg gtt cca caa gga cgc Leu Ala Val Ser Ser Ala Val Pro Thr Ser Trp Val Pro Gln Gly Arg 3300 3305 3310			10054
aca aca tgg tgc att cat ggg aaa ggg gag tgg atg acc acg gaa gac Thr Thr Trp Ser Ile His Gly Lys Gly Glu Trp Met Thr Thr Glu Asp 3315 3320 3325			10102
atg ctt gag gtg tgg aac aga gta tgg ata acc aac aac cca cac atg Met Leu Glu Val Trp Asn Arg Val Trp Ile Thr Asn Asn Pro His Met 3330 3335 3340			10150
cag gac aag aca atg gtg aaa aaa tgg aga gat gtc cct tat cta acc Gln Asp Lys Thr Met Val Lys Lys Trp Arg Asp Val Pro Tyr Leu Thr 3345 3350 3355 3360			10198
aag aga caa gac aag ctg tgc gga tca ctg att gga atg acc aat agg Lys Arg Gln Asp Lys Leu Cys Gly Ser Leu Ile Gly Met Thr Asn Arg 3365 3370 3375			10246
gcc acc tgg gcc tcc cac atc cat tta gtc atc cat cgt atc cga acg Ala Thr Trp Ala Ser His Ile His Leu Val Ile His Arg Ile Arg Thr 3380 3385 3390			10294
ctg att gga cag gag aaa tac act gac tac cta aca gtc atg gac agg			10342

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Leu	Ile	Gly	Gln	Glu	Lys	Tyr	Thr	Asp	Tyr	Leu	Thr	Val	Met	Asp	Arg		
	3395						3400					3405					
tat	tct	gtg	gat	gct	gac	ctg	caa	ctg	ggt	gag	ctt	atc	tgaaacacca			10391	
Tyr	Ser	Val	Asp	Ala	Asp	Leu	Gln	Leu	Gly	Glu	Leu	Ile					
	3410					3415					3420						
tctaacagga	ataaccggga	tacaaaccac	gggtggagaa	ccggactccc	cacaacctga								10451				
aaccgggata	taaaccacgg	ctggagaacc	gggctccgca	cttaaaatga	aacagaaacc								10511				
gggataaaaa	ctacggatgg	agaaccggac	tccacacatt	gagacagaag	aagttgtcag								10571				
cccagaaccc	cacacgagtt	ttgccactgc	taagctgtga	ggcagtcag	gctgggacag								10631				
ccgacctcca	ggttgcgaaa	aacctgggtt	ctgggacctc	ccaccccaga	gtaaaaagaa								10691				
cgagcctcc	gtaccacccc	tcccacgtgg	tggtagaaa	acggggtcta	gaggttagag								10751				
gagaccctcc	agggaaacaaa	tagtgggacc	atattgacgc	cagggaaaga	ccggagtgg								10811				
tctctgcttt	tcctccagag	gtctgtgagc	acagtttgct	caagaataag	cagacctttg								10871				
gatgacaaac	acaaaaccac	t											10892				

&lt;210&gt; SEQ ID NO 13

&lt;211&gt; LENGTH: 3421

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: derived from Japanese Encephalitis virus and Yellow Fever virus

&lt;400&gt; SEQUENCE: 13

Met	Ser	Gly	Arg	Lys	Ala	Gln	Gly	Lys	Thr	Leu	Gly	Val	Asn	Met	Val		
1				5					10					15			
Arg	Arg	Gly	Val	Arg	Ser	Leu	Ser	Asn	Lys	Ile	Lys	Gln	Lys	Thr	Lys		
			20					25					30				
Gln	Ile	Gly	Asn	Arg	Pro	Gly	Pro	Ser	Arg	Gly	Val	Gln	Gly	Phe	Ile		
		35				40						45					
Phe	Phe	Phe	Leu	Phe	Asn	Ile	Leu	Thr	Gly	Lys	Lys	Ile	Thr	Ala	His		
	50				55						60						
Leu	Lys	Arg	Leu	Trp	Lys	Met	Leu	Asp	Pro	Arg	Gln	Gly	Leu	Ala	Val		
65				70				75						80			
Leu	Arg	Lys	Val	Lys	Arg	Val	Val	Ala	Ser	Leu	Met	Arg	Gly	Leu	Ser		
			85					90						95			
Ser	Arg	Lys	Arg	Arg	Ser	His	Asp	Val	Leu	Thr	Val	Gln	Phe	Leu	Ile		
			100				105						110				
Leu	Gly	Met	Leu	Leu	Met	Thr	Gly	Gly	Met	Lys	Leu	Ser	Asn	Phe	Gln		
	115					120						125					
Gly	Lys	Leu	Leu	Met	Thr	Ile	Asn	Asn	Thr	Asp	Ile	Ala	Asp	Val	Ile		
	130				135						140						
Val	Ile	Pro	Thr	Ser	Lys	Gly	Glu	Asn	Arg	Cys	Trp	Val	Arg	Ala	Ile		
145					150				155						160		
Asp	Val	Gly	Tyr	Met	Cys	Glu	Asp	Thr	Ile	Thr	Tyr	Glu	Cys	Pro	Lys		
			165					170					175				
Leu	Thr	Met	Gly	Asn	Asp	Pro	Glu	Asp	Val	Asp	Cys	Trp	Cys	Asp	Asn		
		180						185					190				
Gln	Glu	Val	Tyr	Val	Gln	Tyr	Gly	Arg	Cys	Thr	Arg	Thr	Arg	His	Ser		
	195					200						205					
Lys	Arg	Ser	Arg	Arg	Ser	Val	Ser	Val	Gln	Thr	His	Gly	Glu	Ser	Ser		
	210				215					220							
Leu	Val	Asn	Lys	Lys	Glu	Ala	Trp	Leu	Asp	Ser	Thr	Lys	Ala	Thr	Arg		

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225	230	235	240
Tyr Leu Met Lys Thr Glu Asn Trp Ile Ile Arg Asn Pro Gly Tyr Ala	245	250	255
Phe Leu Ala Ala Val Leu Gly Trp Met Leu Gly Ser Asn Asn Gly Gln	260	265	270
Arg Val Val Phe Thr Ile Leu Leu Leu Val Ala Pro Ala Tyr Ser	275	280	285
Phe Asn Cys Leu Gly Met Gly Asn Arg Asp Phe Ile Glu Gly Ala Ser	290	295	300
Gly Ala Thr Trp Val Asp Leu Val Leu Glu Gly Asp Ser Cys Leu Thr	305	310	315
Ile Met Ala Asn Asp Lys Pro Thr Leu Asp Val Arg Met Ile Asn Ile	325	330	335
Glu Ala Ser Gln Leu Ala Glu Val Arg Ser Tyr Cys Tyr His Ala Ser	340	345	350
Val Thr Asp Ile Ser Thr Val Ala Arg Cys Pro Thr Thr Gly Glu Ala	355	360	365
His Asn Glu Lys Arg Ala Asp Ser Ser Tyr Val Cys Lys Gln Gly Phe	370	375	380
Thr Asp Arg Gly Trp Gly Asn Gly Cys Gly Phe Phe Gly Lys Gly Ser	385	390	395
Ile Asp Thr Cys Ala Lys Phe Ser Cys Thr Ser Lys Ala Ile Gly Arg	405	410	415
Thr Ile Gln Pro Glu Asn Ile Lys Tyr Lys Val Gly Ile Phe Val His	420	425	430
Gly Thr Thr Thr Ser Glu Asn His Gly Asn Tyr Ser Ala Gln Val Gly	435	440	445
Ala Ser Gln Ala Ala Lys Phe Thr Val Thr Pro Asn Ala Pro Ser Val	450	455	460
Ala Leu Lys Leu Gly Asp Tyr Gly Glu Val Thr Leu Asp Cys Glu Pro	465	470	475
Arg Ser Gly Leu Asn Thr Glu Ala Phe Tyr Val Met Thr Val Gly Ser	485	490	495
Lys Ser Phe Leu Val His Arg Glu Trp Phe His Asp Leu Ala Leu Pro	500	505	510
Trp Thr Ser Pro Ser Ser Thr Ala Trp Arg Asn Arg Glu Leu Leu Met	515	520	525
Glu Phe Glu Gly Ala His Ala Thr Lys Gln Ser Val Val Ala Leu Gly	530	535	540
Ser Gln Glu Gly Gly Leu His His Ala Leu Ala Gly Ala Ile Val Val	545	550	555
Glu Tyr Ser Ser Ser Val Met Leu Thr Ser Gly His Leu Lys Cys Arg	565	570	575
Leu Lys Met Asp Lys Leu Ala Leu Lys Gly Thr Thr Tyr Gly Met Cys	580	585	590
Thr Glu Lys Phe Ser Phe Ala Lys Asn Pro Val Asp Thr Gly His Gly	595	600	605
Thr Val Val Ile Glu Leu Ser Tyr Ser Gly Ser Asp Gly Pro Cys Lys	610	615	620
Ile Pro Ile Val Ser Val Ala Ser Leu Asn Asp Met Thr Pro Val Gly	625	630	635
Arg Leu Val Thr Val Asn Pro Phe Val Ala Thr Ser Ser Ala Asn Ser	645	650	655



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Lys Val Leu Val Glu Met Glu Pro Pro Phe Gly Asp Ser Tyr Ile Val  
 660 665 670  
 Val Gly Arg Gly Asp Lys Gln Ile Asn His His Trp His Lys Ala Gly  
 675 680 685  
 Ser Thr Leu Gly Lys Ala Phe Ser Thr Thr Leu Lys Gly Ala Gln Arg  
 690 695 700  
 Leu Ala Ala Leu Gly Asp Thr Ala Trp Asp Phe Gly Ser Ile Gly Gly  
 705 710 715 720  
 Val Phe Asn Ser Ile Gly Arg Ala Val His Gln Val Phe Gly Gly Ala  
 725 730 735  
 Phe Arg Thr Leu Phe Gly Gly Met Ser Trp Ile Thr Gln Gly Leu Met  
 740 745 750  
 Gly Ala Leu Leu Leu Trp Met Gly Val Asn Ala Arg Asp Arg Ser Ile  
 755 760 765  
 Ala Leu Ala Phe Leu Ala Thr Gly Gly Val Leu Val Phe Leu Ala Thr  
 770 775 780  
 Asn Val Gly Ala Asp Gln Gly Cys Ala Ile Asn Phe Gly Lys Arg Glu  
 785 790 795 800  
 Leu Lys Cys Gly Asp Gly Ile Phe Ile Phe Arg Asp Ser Asp Asp Trp  
 805 810 815  
 Leu Asn Lys Tyr Ser Tyr Tyr Pro Glu Asp Pro Val Lys Leu Ala Ser  
 820 825 830  
 Ile Val Lys Ala Ser Phe Glu Glu Gly Lys Cys Gly Leu Asn Ser Val  
 835 840 845  
 Asp Ser Leu Glu His Glu Met Trp Arg Ser Arg Ala Asp Glu Ile Asn  
 850 855 860  
 Ala Ile Phe Glu Glu Asn Glu Val Asp Ile Ser Val Val Val Gln Asp  
 865 870 875 880  
 Pro Lys Asn Val Tyr Gln Arg Gly Thr His Pro Phe Ser Arg Ile Arg  
 885 890 895  
 Asp Gly Leu Gln Tyr Gly Trp Lys Thr Trp Gly Lys Asn Leu Val Phe  
 900 905 910  
 Ser Pro Gly Arg Lys Asn Gly Ser Phe Ile Ile Asp Gly Lys Ser Arg  
 915 920 925  
 Lys Glu Cys Pro Phe Ser Asn Arg Val Trp Asn Ser Phe Gln Ile Glu  
 930 935 940  
 Glu Phe Gly Thr Gly Val Phe Thr Thr Arg Val Tyr Met Asp Ala Val  
 945 950 955 960  
 Phe Glu Tyr Thr Ile Asp Cys Asp Gly Ser Ile Leu Gly Ala Ala Val  
 965 970 975  
 Asn Gly Lys Lys Ser Ala His Gly Ser Pro Thr Phe Trp Met Gly Ser  
 980 985 990  
 His Glu Val Asn Gly Thr Trp Met Ile His Thr Leu Glu Ala Leu Asp  
 995 1000 1005  
 Tyr Lys Glu Cys Glu Trp Pro Leu Thr His Thr Ile Gly Thr Ser Val  
 1010 1015 1020  
 Glu Glu Ser Glu Met Phe Met Pro Arg Ser Ile Gly Gly Pro Val Ser  
 1025 1030 1035 1040  
 Ser His Asn His Ile Pro Gly Tyr Lys Val Gln Thr Asn Gly Pro Trp  
 1045 1050 1055  
 Met Gln Val Pro Leu Glu Val Lys Arg Glu Ala Cys Pro Gly Thr Ser  
 1060 1065 1070



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1490	1495	1500
Pro Lys Ile Ile Glu Glu Cys Glu His Leu Glu Asp Gly Ile Tyr Gly 1505 1510 1515 1520		
Ile Phe Gln Ser Thr Phe Leu Gly Ala Ser Gln Arg Gly Val Gly Val 1525 1530 1535		
Ala Gln Gly Gly Val Phe His Thr Met Trp His Val Thr Arg Gly Ala 1540 1545 1550		
Phe Leu Val Arg Asn Gly Lys Lys Leu Ile Pro Ser Trp Ala Ser Val 1555 1560 1565		
Lys Glu Asp Leu Val Ala Tyr Gly Gly Ser Trp Lys Leu Glu Gly Arg 1570 1575 1580		
Trp Asp Gly Glu Glu Glu Val Gln Leu Ile Ala Ala Val Pro Gly Lys 1585 1590 1595 1600		
Asn Val Val Asn Val Gln Thr Lys Pro Ser Leu Phe Lys Val Arg Asn 1605 1610 1615		
Gly Gly Glu Ile Gly Ala Val Ala Leu Asp Tyr Pro Ser Gly Thr Ser 1620 1625 1630		
Gly Ser Pro Ile Val Asn Arg Asn Gly Glu Val Ile Gly Leu Tyr Gly 1635 1640 1645		
Asn Gly Ile Leu Val Gly Asp Asn Ser Phe Val Ser Ala Ile Ser Gln 1650 1655 1660		
Thr Glu Val Lys Glu Glu Gly Lys Glu Glu Leu Gln Glu Ile Pro Thr 1665 1670 1675 1680		
Met Leu Lys Lys Gly Met Thr Thr Val Leu Asp Phe His Pro Gly Ala 1685 1690 1695		
Gly Lys Thr Arg Arg Phe Leu Pro Gln Ile Leu Ala Glu Cys Ala Arg 1700 1705 1710		
Arg Arg Leu Arg Thr Leu Val Leu Ala Pro Thr Arg Val Val Leu Ser 1715 1720 1725		
Glu Met Lys Glu Ala Phe His Gly Leu Asp Val Lys Phe His Thr Gln 1730 1735 1740		
Ala Phe Ser Ala His Gly Ser Gly Arg Glu Val Ile Asp Ala Met Cys 1745 1750 1755 1760		
His Ala Thr Leu Thr Tyr Arg Met Leu Glu Pro Thr Arg Val Val Asn 1765 1770 1775		
Trp Glu Val Ile Ile Met Asp Glu Ala His Phe Leu Asp Pro Ala Ser 1780 1785 1790		
Ile Ala Ala Arg Gly Trp Ala Ala His Arg Ala Arg Ala Asn Glu Ser 1795 1800 1805		
Ala Thr Ile Leu Met Thr Ala Thr Pro Pro Gly Thr Ser Asp Glu Phe 1810 1815 1820		
Pro His Ser Asn Gly Glu Ile Glu Asp Val Gln Thr Asp Ile Pro Ser 1825 1830 1835 1840		
Glu Pro Trp Asn Thr Gly His Asp Trp Ile Leu Ala Asp Lys Arg Pro 1845 1850 1855		
Thr Ala Trp Phe Leu Pro Ser Ile Arg Ala Ala Asn Val Met Ala Ala 1860 1865 1870		
Ser Leu Arg Lys Ala Gly Lys Ser Val Val Val Leu Asn Arg Lys Thr 1875 1880 1885		
Phe Glu Arg Glu Tyr Pro Thr Ile Lys Gln Lys Lys Pro Asp Phe Ile 1890 1895 1900		
Leu Ala Thr Asp Ile Ala Glu Met Gly Ala Asn Leu Cys Val Glu Arg 1905 1910 1915 1920		

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Val Leu Asp Cys Arg Thr Ala Phe Lys Pro Val Leu Val Asp Glu Gly  
 1925 1930 1935  
 Arg Lys Val Ala Ile Lys Gly Pro Leu Arg Ile Ser Ala Ser Ser Ala  
 1940 1945 1950  
 Ala Gln Arg Arg Gly Arg Ile Gly Arg Asn Pro Asn Arg Asp Gly Asp  
 1955 1960 1965  
 Ser Tyr Tyr Tyr Ser Glu Pro Thr Ser Glu Asn Asn Ala His His Val  
 1970 1975 1980  
 Cys Trp Leu Glu Ala Ser Met Leu Leu Asp Asn Met Glu Val Arg Gly  
 1985 1990 1995 2000  
 Gly Met Val Ala Pro Leu Tyr Gly Val Glu Gly Thr Lys Thr Pro Val  
 2005 2010 2015  
 Ser Pro Gly Glu Met Arg Leu Arg Asp Asp Gln Arg Lys Val Phe Arg  
 2020 2025 2030  
 Glu Leu Val Arg Asn Cys Asp Leu Pro Val Trp Leu Ser Trp Gln Val  
 2035 2040 2045  
 Ala Lys Ala Gly Leu Lys Thr Asn Asp Arg Lys Trp Cys Phe Glu Gly  
 2050 2055 2060  
 Pro Glu Glu His Glu Ile Leu Asn Asp Ser Gly Glu Thr Val Lys Cys  
 2065 2070 2075 2080  
 Arg Ala Pro Gly Gly Ala Lys Lys Pro Leu Arg Pro Arg Trp Cys Asp  
 2085 2090 2095  
 Glu Arg Val Ser Ser Asp Gln Ser Ala Leu Ser Glu Phe Ile Lys Phe  
 2100 2105 2110  
 Ala Glu Gly Arg Arg Gly Ala Ala Glu Val Leu Val Val Leu Ser Glu  
 2115 2120 2125  
 Leu Pro Asp Phe Leu Ala Lys Lys Gly Gly Glu Ala Met Asp Thr Ile  
 2130 2135 2140  
 Ser Val Phe Leu His Ser Glu Glu Gly Ser Arg Ala Tyr Arg Asn Ala  
 2145 2150 2155 2160  
 Leu Ser Met Met Pro Glu Ala Met Thr Ile Val Met Leu Phe Ile Leu  
 2165 2170 2175  
 Ala Gly Leu Leu Thr Ser Gly Met Val Ile Phe Phe Met Ser Pro Lys  
 2180 2185 2190  
 Gly Ile Ser Arg Met Ser Met Ala Met Gly Thr Met Ala Gly Cys Gly  
 2195 2200 2205  
 Tyr Leu Met Phe Leu Gly Gly Val Lys Pro Thr His Ile Ser Tyr Val  
 2210 2215 2220  
 Met Leu Ile Phe Phe Val Leu Met Val Val Val Ile Pro Glu Pro Gly  
 2225 2230 2235 2240  
 Gln Gln Arg Ser Ile Gln Asp Asn Gln Val Ala Tyr Leu Ile Ile Gly  
 2245 2250 2255  
 Ile Leu Thr Leu Val Ser Ala Val Ala Ala Asn Glu Leu Gly Met Leu  
 2260 2265 2270  
 Glu Lys Thr Lys Glu Asp Leu Phe Gly Lys Lys Asn Leu Ile Pro Ser  
 2275 2280 2285  
 Ser Ala Ser Pro Trp Ser Trp Pro Asp Leu Asp Leu Lys Pro Gly Ala  
 2290 2295 2300  
 Ala Trp Thr Val Tyr Val Gly Ile Val Thr Met Leu Ser Pro Met Leu  
 2305 2310 2315 2320  
 His His Trp Ile Lys Val Glu Tyr Gly Asn Leu Ser Leu Ser Gly Ile  
 2325 2330 2335

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Ala	Gln	Ser	Ala	Ser	Val	Leu	Ser	Phe	Met	Asp	Lys	Gly	Ile	Pro	Phe		
			2340					2345					2350				
Met	Lys	Met	Asn	Ile	Ser	Val	Ile	Met	Leu	Leu	Val	Ser	Gly	Trp	Asn		
		2355					2360						2365				
Ser	Ile	Thr	Val	Met	Pro	Leu	Leu	Cys	Gly	Ile	Gly	Cys	Ala	Met	Leu		
		2370				2375						2380					
His	Trp	Ser	Leu	Ile	Leu	Pro	Gly	Ile	Lys	Ala	Gln	Gln	Ser	Lys	Leu		
	2385				2390					2395					2400		
Ala	Gln	Arg	Arg	Val	Phe	His	Gly	Val	Ala	Lys	Asn	Pro	Val	Val	Asp		
				2405					2410						2415		
Gly	Asn	Pro	Thr	Val	Asp	Ile	Glu	Glu	Ala	Pro	Glu	Met	Pro	Ala	Leu		
			2420					2425						2430			
Tyr	Glu	Lys	Lys	Leu	Ala	Leu	Tyr	Leu	Leu	Leu	Ala	Leu	Ser	Leu	Ala		
	2435						2440							2445			
Ser	Val	Ala	Met	Cys	Arg	Thr	Pro	Phe	Ser	Leu	Ala	Glu	Gly	Ile	Val		
	2450					2455						2460					
Leu	Ala	Ser	Ala	Ala	Leu	Gly	Pro	Leu	Ile	Glu	Gly	Asn	Thr	Ser	Leu		
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Leu	Trp	Asn	Gly	Pro	Met	Ala	Val	Ser	Met	Thr	Gly	Val	Met	Arg	Gly		
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Asn	His	Tyr	Ala	Phe	Val	Gly	Val	Met	Tyr	Asn	Leu	Trp	Lys	Met	Lys		
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Thr	Gly	Arg	Arg	Gly	Ser	Ala	Asn	Gly	Lys	Thr	Leu	Gly	Glu	Val	Trp		
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Lys	Arg	Glu	Leu	Asn	Leu	Leu	Asp	Lys	Arg	Gln	Phe	Glu	Leu	Tyr	Lys		
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Arg	Thr	Asp	Ile	Val	Glu	Val	Asp	Arg	Asp	Thr	Ala	Arg	Arg	His	Leu		
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Ala	Glu	Gly	Lys	Val	Asp	Thr	Gly	Val	Ala	Val	Ser	Arg	Gly	Thr	Ala		
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Val	Ile	Asp	Leu	Gly	Cys	Gly	Arg	Gly	Gly	Trp	Cys	Tyr	Tyr	Ala	Ala		
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Gly	His	Glu	Lys	Pro	Met	Asn	Val	Gln	Ser	Leu	Gly	Trp	Asn	Ile	Ile		
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Thr	Phe	Lys	Asp	Lys	Thr	Asp	Ile	His	Arg	Leu	Glu	Pro	Val	Lys	Cys		
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Asp	Thr	Leu	Leu	Cys	Asp	Ile	Gly	Glu	Ser	Ser	Ser	Ser	Ser	Val	Thr		
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Glu	Gly	Glu	Arg	Thr	Val	Arg	Val	Leu	Asp	Thr	Val	Glu	Lys	Trp	Leu		
		2675					2680						2685				
Ala	Cys	Gly	Val	Asp	Asn	Phe	Cys	Val	Lys	Val	Leu	Ala	Pro	Tyr	Met		
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Pro	Asp	Val	Leu	Glu	Lys	Leu	Glu	Leu	Leu	Gln	Arg	Arg	Phe	Gly	Gly		
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Thr	Val	Ile	Arg	Asn	Pro	Leu	Ser	Arg	Asn	Ser	Thr	His	Glu	Met	Tyr		
			2725						2730					2735			
Tyr	Val	Ser	Gly	Ala	Arg	Ser	Asn	Val	Thr	Phe	Thr	Val	Asn	Gln	Thr		
		2740							2745					2750			
Ser	Arg	Leu	Leu	Met	Arg	Arg	Met	Arg	Arg	Pro	Thr	Gly	Lys	Val	Thr		

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Leu Glu Ala Asp Val Ile Leu Pro Ile Gly Thr Arg Ser Val Glu Thr 2770	2775	2780
Asp Lys Gly Pro Leu Asp Lys Glu Ala Ile Glu Glu Arg Val Glu Arg 2785	2790	2800
Ile Lys Ser Glu Tyr Met Thr Ser Trp Phe Tyr Asp Asn Asp Asn Pro 2805	2810	2815
Tyr Arg Thr Trp His Tyr Cys Gly Ser Tyr Val Thr Lys Thr Ser Gly 2820	2825	2830
Ser Ala Ala Ser Met Val Asn Gly Val Ile Lys Ile Leu Thr Tyr Pro 2835	2840	2845
Trp Asp Arg Ile Glu Glu Val Thr Arg Met Ala Met Thr Asp Thr Thr 2850	2855	2860
Pro Phe Gly Gln Gln Arg Val Phe Lys Glu Lys Val Asp Thr Arg Ala 2865	2870	2875
Lys Asp Pro Pro Ala Gly Thr Arg Lys Ile Met Lys Val Val Asn Arg 2885	2890	2895
Trp Leu Phe Arg His Leu Ala Arg Glu Lys Asn Pro Arg Leu Cys Thr 2900	2905	2910
Lys Glu Glu Phe Ile Ala Lys Val Arg Ser His Ala Ala Ile Gly Ala 2915	2920	2925
Tyr Leu Glu Glu Gln Glu Trp Lys Thr Ala Asn Glu Ala Val Gln 2930	2935	2940
Asp Pro Lys Phe Trp Glu Leu Val Asp Glu Glu Arg Lys Leu His Gln 2945	2950	2955
Gln Gly Arg Cys Arg Thr Cys Val Tyr Asn Met Met Gly Lys Arg Glu 2965	2970	2975
Lys Lys Leu Ser Glu Phe Gly Lys Ala Lys Gly Ser Arg Ala Ile Trp 2980	2985	2990
Tyr Met Trp Leu Gly Ala Arg Tyr Leu Glu Phe Glu Ala Leu Gly Phe 2995	3000	3005
Leu Asn Glu Asp His Trp Ala Ser Arg Glu Asn Ser Gly Gly Gly Val 3010	3015	3020
Glu Gly Ile Gly Leu Gln Tyr Leu Gly Tyr Val Ile Arg Asp Leu Ala 3025	3030	3035
Ala Met Asp Gly Gly Gly Phe Tyr Ala Asp Asp Thr Ala Gly Trp Asp 3045	3050	3055
Thr Arg Ile Thr Glu Ala Asp Leu Asp Asp Glu Gln Glu Ile Leu Asn 3060	3065	3070
Tyr Met Ser Pro His His Lys Lys Leu Ala Gln Ala Val Met Glu Met 3075	3080	3085
Thr Tyr Lys Asn Lys Val Val Lys Val Leu Arg Pro Ala Pro Gly Gly 3090	3095	3100
Lys Ala Tyr Met Asp Val Ile Ser Arg Arg Asp Gln Arg Gly Ser Gly 3105	3110	3115
Gln Val Val Thr Tyr Ala Leu Asn Thr Ile Thr Asn Leu Lys Val Gln 3125	3130	3135
Leu Ile Arg Met Ala Glu Ala Glu Met Val Ile His His Gln His Val 3140	3145	3150
Gln Asp Cys Asp Glu Ser Val Leu Thr Arg Leu Glu Ala Trp Leu Thr 3155	3160	3165
Glu His Gly Cys Asp Arg Leu Lys Arg Met Ala Val Ser Gly Asp Asp 3170	3175	3180

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Cys Val Val Arg Pro Ile Asp Asp Arg Phe Gly Leu Ala Leu Ser His  
 3185 3190 3195 3200  
 Leu Asn Ala Met Ser Lys Val Arg Lys Asp Ile Ser Glu Trp Gln Pro  
 3205 3210 3215  
 Ser Lys Gly Trp Asn Asp Trp Glu Asn Val Pro Phe Cys Ser His His  
 3220 3225 3230  
 Phe His Glu Leu Gln Leu Lys Asp Gly Arg Arg Ile Val Val Pro Cys  
 3235 3240 3245  
 Arg Glu Gln Asp Glu Leu Ile Gly Arg Gly Arg Val Ser Pro Gly Asn  
 3250 3255 3260  
 Gly Trp Met Ile Lys Glu Thr Ala Cys Leu Ser Lys Ala Tyr Ala Asn  
 3265 3270 3275 3280  
 Met Trp Ser Leu Met Tyr Phe His Lys Arg Asp Met Arg Leu Leu Ser  
 3285 3290 3295  
 Leu Ala Val Ser Ser Ala Val Pro Thr Ser Trp Val Pro Gln Gly Arg  
 3300 3305 3310  
 Thr Thr Trp Ser Ile His Gly Lys Gly Glu Trp Met Thr Thr Glu Asp  
 3315 3320 3325  
 Met Leu Glu Val Trp Asn Arg Val Trp Ile Thr Asn Asn Pro His Met  
 3330 3335 3340  
 Gln Asp Lys Thr Met Val Lys Lys Trp Arg Asp Val Pro Tyr Leu Thr  
 3345 3350 3355 3360  
 Lys Arg Gln Asp Lys Leu Cys Gly Ser Leu Ile Gly Met Thr Asn Arg  
 3365 3370 3375  
 Ala Thr Trp Ala Ser His Ile His Leu Val Ile His Arg Ile Arg Thr  
 3380 3385 3390  
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21

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25

<210> SEQ ID NO 16  
 <211> LENGTH: 21  
 <212> TYPE: DNA  
 <213> ORGANISM: Artificial Sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: Derived from Yellow Fever virus and Dengue-1

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## virus

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&lt;210&gt; SEQ ID NO 17

&lt;211&gt; LENGTH: 26

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Derived from Yellow Fever virus and Dengue-1 virus

&lt;400&gt; SEQUENCE: 17

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26

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&lt;211&gt; LENGTH: 21

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Derived from Yellow Fever virus and Dengue-2 virus

&lt;400&gt; SEQUENCE: 18

aaggtagatt ggtgtgcatt g

21

&lt;210&gt; SEQ ID NO 19

&lt;211&gt; LENGTH: 26

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Derived from Yellow Fever virus and Dengue-2 virus

&lt;400&gt; SEQUENCE: 19

aaccctcagt accaccgcg gtttaa

26

&lt;210&gt; SEQ ID NO 20

&lt;211&gt; LENGTH: 21

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Derived from Yellow Fever virus and Dengue-3 virus

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&lt;210&gt; SEQ ID NO 21

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&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Derived from Yellow Fever virus and Dengue-3 virus

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acccccagca ccaccgcg gtttaa

25

&lt;210&gt; SEQ ID NO 22

&lt;211&gt; LENGTH: 21

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Derived from Yellow Fever virus and Dengue-4 virus



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&lt;210&gt; SEQ ID NO 23

&lt;211&gt; LENGTH: 25

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&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Derived from Yellow Fever virus and Dengue-4 virus

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&lt;211&gt; LENGTH: 21

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&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Derived from Yellow Fever virus and St. Louis Encephalitis virus

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&lt;210&gt; SEQ ID NO 25

&lt;211&gt; LENGTH: 25

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Derived from Yellow Fever virus and St. Louis Encephalitis virus

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&lt;210&gt; SEQ ID NO 26

&lt;211&gt; LENGTH: 21

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Derived from Yellow Fever virus and Murray Valley Encephalitis virus

&lt;400&gt; SEQUENCE: 26

aatttcgaaa ggtggaaggt c

21

&lt;210&gt; SEQ ID NO 27

&lt;211&gt; LENGTH: 26

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Derived from Yellow Fever virus and Murray Valley Encephalitis virus

&lt;400&gt; SEQUENCE: 27

gaccggtggt tacagccgcg gtttaa

26

&lt;210&gt; SEQ ID NO 28

&lt;211&gt; LENGTH: 21

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Derived from Yellow Fever virus and Tick-Borne Encephalitis virus

-continued

&lt;400&gt; SEQUENCE: 28

tactgcgaac gacgttgcca c

21

&lt;210&gt; SEQ ID NO 29

&lt;211&gt; LENGTH: 25

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Derived from Yellow Fever virus and Tick-Borne Encephalitis virus

&lt;400&gt; SEQUENCE: 29

actgggaacc tcaccgcgg tttaa

25

What is claimed is:

1. A chimeric live, infectious, attenuated virus, comprising:

a yellow fever virus in which the nucleotide sequence encoding a prM-E protein is either deleted, truncated, or mutated so that functional yellow fever virus prM-E protein is not expressed, and

integrated into the genome of said yellow fever virus, a nucleotide sequence encoding a prM-E protein of a second, different flavivirus, to that said prM-E protein of said second flavivirus is expressed, wherein the capsid protein of said chimeric virus is from yellow fever virus.

2. The chimeric virus of claim 1, wherein said second flavivirus is a Japanese Encephalitis (JE) virus.

3. The chimeric virus of claim 1, wherein the nucleotide sequence encoding the prM-E protein of said, second, different flavivirus replaces the nucleotide sequence encoding the prM-E protein of said yellow fever virus.

4. The chimeric virus of claim 1, wherein said nucleotide sequence encoding said prM-E protein of said second, different flavivirus comprises a mutation that prevents prM cleavage to produce M protein.

5. The chimeric virus of claim 1, wherein the NS2B-3-protease recognition site and the signal sequences and cleavage sites at the C/prM and E/NS1 junctions are maintained in construction of said chimeric virus.

6. The chimeric virus of claim 1, wherein said second flavivirus is a Murray Valley Encephalitis virus.

7. The chimeric virus of claim 1, wherein said second flavivirus is a St. Louis Encephalitis virus.

8. The chimeric virus of claim 1, wherein said second flavivirus is a West Nile virus.

9. The chimeric virus of claim 1, wherein said second flavivirus is a Tick-borne Encephalitis virus.

10. The chimeric virus of claim 1, wherein the signal sequence at the C/prM junction is maintained in construction of said chimeric virus.

11. A method of preventing or treating Japanese encephalitis virus infection in a patient, said method comprising administering to said patient a chimeric, live, infectious, attenuated virus comprising:

a yellow fever virus in which the nucleotide sequence encoding a prM-E protein is either deleted, truncated, or mutated so that functional yellow fever virus prM-E protein is not expressed, and

integrated into the genome of said yellow fever virus, a nucleotide sequence encoding a prM-E protein of Japanese encephalitis virus strain SA-14-14-2 or Japanese encephalitis virus strain Nakayama, wherein the capsid protein of said chimeric virus is from yellow fever virus.

12. The method of claim 11, wherein the nucleotide sequence encoding the prM-E protein of said Japanese encephalitis virus replaces the nucleotide sequence encoding the prM-E protein of said yellow fever virus.

13. The method of claim 11, wherein said nucleotide sequence encoding said prM-E protein of said Japanese encephalitis virus comprises a mutation that prevents prM cleavage to produce M protein.

14. The method of claim 11, wherein the NS2B-3 protease recognition site and the signal sequences and cleavage sites at the C/prM and E/NS1 junctions are maintained in connection of said chimeric virus.

\* \* \* \* \*

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